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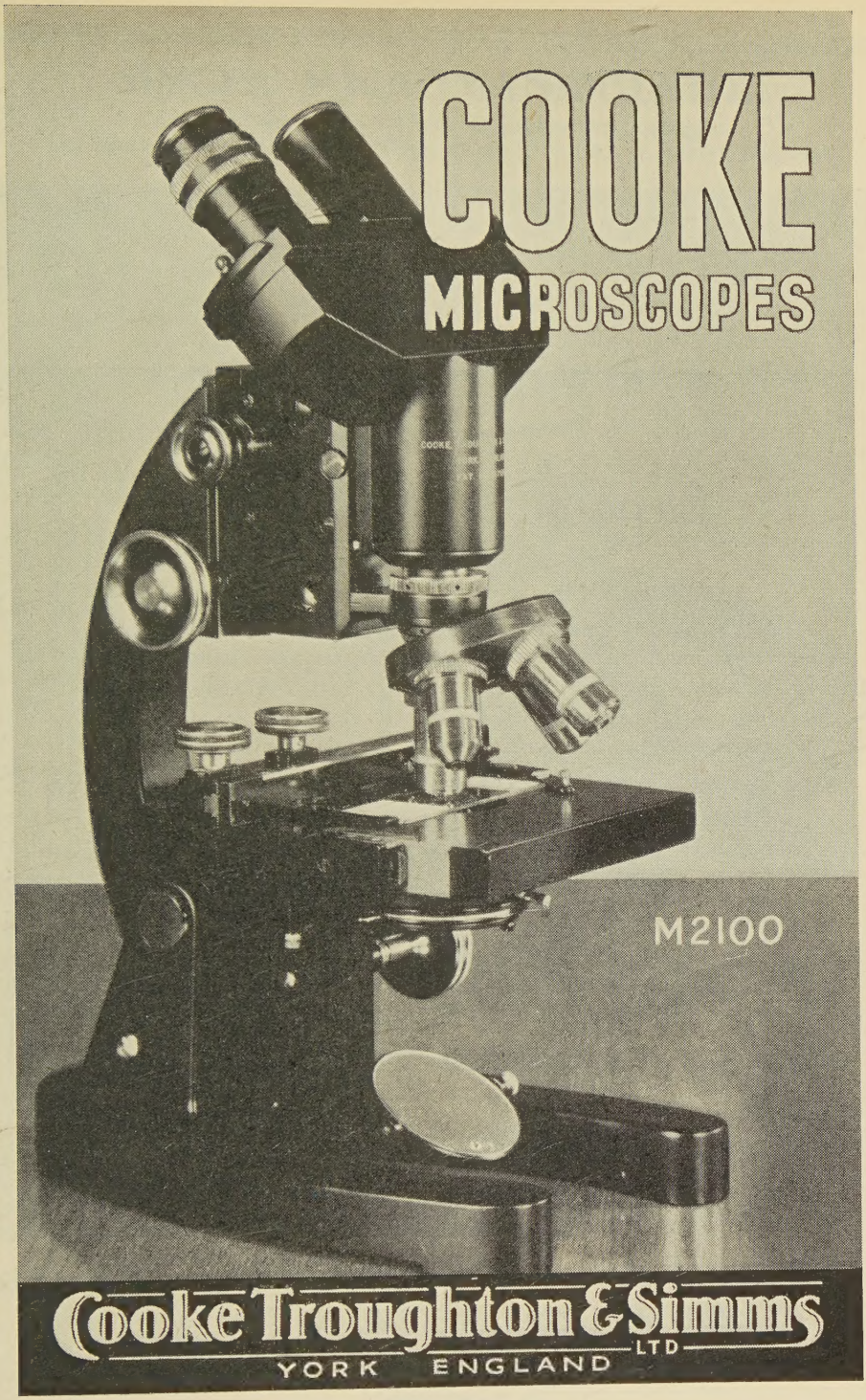
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On the Functional Morphology of the Alimentary Tract of Some Fish in Relation to Differences in their Feeding Habits: Anatomy and Histology

BY

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With Three Plates

CONTENTS

INTRODUCTION

THE present work is the continuation of a series of studies (Al-Hussaini, 1945–7) attempting to correlate the structure of the alimentary canal with the feeding habits of teleosts. These earlier studies were based on distantly related species and thus the differences observed and described might well be due to congenital factors rather than to purely adaptive causes—of this no certain answer can yet be given. The present study is an attempt to exclude congenital factors as nearly as possible by choosing three closely allied species

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with different feeding habits, namely, the mirror (king or spiegel) carp (*Cyprinus carpio* L.), the roach (*Rutilus rutilus* (L.)), and the gudgeon (*Gobio gobio* (L.)), species which will hereafter be referred to by their generic names only. From the point of view of the general problem this series suffers from a slight defect in that all three are capable of dealing with a mixed diet, that is, they are strictly speaking omnivorous. Nevertheless, the mirror carp (similar in feeding habits to the common carp) ingests more plant than animal food (Couch, 1865; Day, 1880–4; Regan, 1911), in the gudgeon animal food preponderates in the diet (Yarrell, 1841; Day, 1880–4; Šusta, 1888; Regan, 1911, Hartley, 1940, 1947), while the roach is omnivorous in the true sense (Grevé, 1897; Regan, 1911; Hartley, 1940, 1947), and they have been customarily described as herbivorous, carnivorous, and omnivorous feeders according to the material which composes the greater bulk of their diet. An attempt was made to justify these conclusions experimentally, but unfortunately, owing to unavoidable circumstances, the number of fish which could eventually be spared was very small, so that not more than some three or four individuals could be fed on any one specialized diet. Such results as were obtained, however, supported the view above expressed. It may be noted further that the carp and the gudgeon both seek their food from the bottom.

The present account deals simply with the comparative anatomy and histology of the mouth, pharynx, and gut of the three types. This will be followed by an account of the cytological, histochemical, and physiological aspects of the problem so that the 'functional morphology' and the adaptive correlations may thereby acquire a firmer basis.

Historical Survey

Good historical reviews of early work have been written by Oppel (1896), Sullivan (1907), and Biedermann (1911), and it is not proposed to add much to them beyond what is necessary to provide an appropriate background for the present study. Amongst the more important works that have appeared since these reviews may be mentioned Jacobshagen (1911, 1913, 1915, 1937), who extended Eggeling's (1907) earlier study of the detailed configuration of the intestinal mucosa, from both taxonomic and adaptive points of view, while Pictet (1909) working on similar lines studied, in particular, the gut mucosa of five Cyprinid species. Dawes (1929) described the histology of the gut of the plaice and defined the pharynx, oesophagus, and rectum, pointing out certain changes in both cytoplasm and nucleus of the columnar epithelial cells which he associated with secretory activity. Of the more recent workers directly concerned with Cyprinids, mention must be made of Rogick (1931) working on *Campostoma anomalum*, and of Curry (1939) who studied the common carp—*Cyprinus carpio communis*. Sarbahi (1940) differentiated a caecal from a pyloric portion of the intestinal bulb and described conical cells which hang freely into the lumen of the rectum in *Labeo rohita*. McVay and Kaan (1940) investigated the goldfish—*Carassius auratus*—and discerned the changes which occur in the cells of the intestinal epithelium, already

referred to by Dawes, but offered no explanation of the phenomenon. Finally, Klust (1940) described the changes which occur as development proceeds in several Cyprinid species. Authors who have attempted to correlate the structure of the alimentary tract with the feeding habits of the fish have already been reviewed in a previous paper (cf. Al-Hussaini, 1945).

Material and Methods

This work was carried out in the Department of Zoology, Sheffield University, the roach and gudgeon being supplied by the Sheffield Corporation Water Works from their ponds surrounding the city, while the mirror carp came from the Surrey Trout Farm (Haslemere). The Red Sea types used for comparison were collected personally from the neighbourhood of the Marine Biological Station at Ghardaqa, while other supplementary types came from various English waters.

The techniques employed are numerous, and it will be more convenient to refer to them in the appropriate sections of the text with which they are individually concerned. When an anaesthetic was needed for experimental work urethane was used, while the fishes were normally killed by a blow on the head.

In the following account the omnivorous roach is described as the central type and then compared with the other two species.

The Anatomy of the Alimentary Tract

The Protractile Apparatus of the Mouth

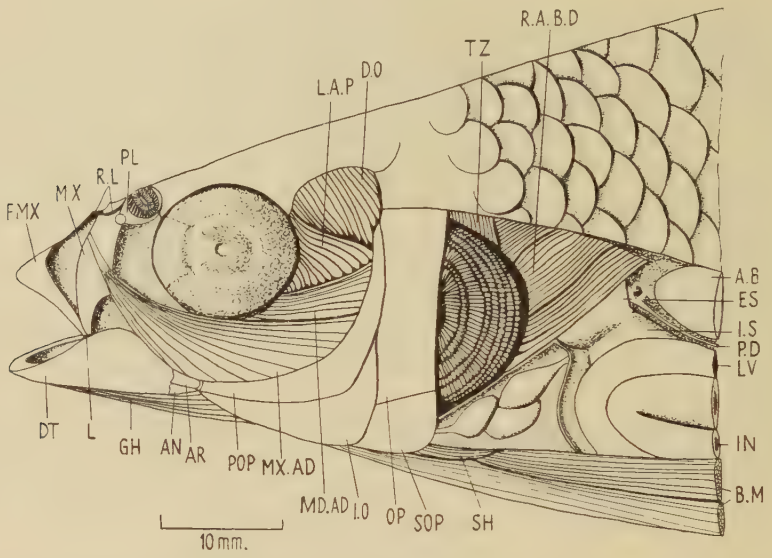
The mouth of *Rutilus* (Text-fig. 1) is small and agrees with the 'normal' type of mouth described by Gregory (1933) in *Micropterus*, except that in the *Rutilus* both jaws lack teeth. Mouths of this type have a moderate gape and moderate protrusibility. The maxillae are excluded from the gape and the premaxillae develop articular and ascending processes.

This latter process, or 'spine', is attached to the ethmoid by the rostral ligament (R.L.) containing a small cartilaginous nodule. According to Eaton (1935), this nodule ensures that the ligament shall bend in precisely the same way each time the jaw retracts. The protrusibility of the mouth depends largely on the length of the ligament which both allows, and checks, the forward thrusting of the upper jaw, and also on the length of the premaxillary spine; the larger the spine the more protrusible the mouth (Eaton, 1943).

The ventral end of the maxilla (MX.) is attached to the premaxilla by a short flexible ligament near the angle of the mouth. Dorsally the two maxillae meet and form a groove in which the premaxillary spine and ligament slide back and forth. The maxillae are also loosely attached to the vomer and palatine, so that when premaxillae are thrust forwards the maxillae do not impede but rather assist this process, since this dorsal maxillary hinge allows their ventral ends to thrust forwards with the premaxillae.

In the lower jaw the dentaries are held together at the symphysis by a very short ligament allowing a slight amount of movement.

The advantage of a protrusible mouth to *Rutilus* is that it increases the range over which the fish can take food as well as enabling it to engulf small prey or other food particles entire by a quick snap, and thus compensates for the absence of teeth on both jaws and palate.



TEXT-FIG. 1. Lateral view of head of *R. rutilus* showing protraction of the mouth. The lacrymal, the posterior part of the operculum with its branchiostegal rays, and part of the pectoral girdle have been removed, and the anterior part of the body cavity exposed.

In *Gobio* the mouth is larger than in *Rutilus*, and a barb is attached to each jaw angle. The upper jaw is larger than the lower so that the mouth opening is directed distinctly downwards. Both the premaxillary spine and the rostral ligament are longer and hence the mouth is more protrusible than in *Rutilus*. When the jaws are fully protruded the mouth opening is completely ventral, a feature obviously connected with the bottom-feeding habit of the fish. When roach and gudgeon were kept together the roach would immediately dash at any food (e.g. insects) thrown in, but the gudgeon ignored it completely until some had sunk to the bottom of the tank. The differences in the shape of the mouth and in the length of its constituent parts represent, therefore, the morphological expression of differences of feeding habits.

In the herbivorous *Cyprinus* the head is relatively larger in proportion to the rest of the body than in either of the other two species. The length of the rostral ligament and premaxillary spine, and hence the protrusibility of the mouth, is intermediate between *Rutilus* and *Gobio*. There are four barbs,

shorter than those of *Gobio*, around the mouth, one from each jaw angle and a dorsal pair from the skin covering the maxillae.

Although *Cyprinus* feeds more easily from the bottom, yet it can rise to take food from the surface and hence combines the feeding habits of *Rutilus* and *Gobio*. These differences in feeding habits are also reflected in other structures. Thus Evans (1940) showed that in *Rutilus* the eyes and optic lobes are much larger than in either *Gobio* or *Cyprinus*, but in the latter two species the facial and vagal lobes (concerned with taste) are much better developed than in *Rutilus*. Thus *Rutilus*, with its superior vision, is able to snap its food freely in the water while *Gobio* and *Cyprinus* normally seek it from the bottom and have developed barbs richly supplied with taste-buds for this purpose (cf. p. 126).

The Pharyngeal Dental Apparatus

The fifth branchial arch is reduced to a single strong bone on each side, the 'os pharyngeus inferior' (Goodrich, 1930, p. 440), usually referred to in cyprinids as the 'pharyngeal jaw'. The modified arch in *Rutilus* (Pl. II, fig. 7*a*, *b*, *c*) is typical of the type described as 'omnivorous' by Chu (1935) in Chinese cyprinids. Each half of the complete arch is about three times as long as it is broad, its posterior limb is about one and a half times as long as the anterior, while at the angle between them there is a distinct lateral projection just behind the second tooth. The posterior surface is pitted (number of pits variable) and wing-like. An edentulous process curves strongly dorsally, making an obtuse posterior angle. This process is compressed and sometimes tapering. It is directed medially, reaching the otic region of the skull where it meets its fellow from the opposite side; thus the two processes form an arch bounding the opening to the oesophagus.

In *Gobio* (Pl. II, fig. 7*f*) the length of each pharyngeal bone is about four and a half times the width, the posterior limb is not longer than the anterior limb, while the pitted surface is narrower than in *Rutilus*, dimensions which agree with those given by Chu (1935) for the carnivorous species of the subfamily Gobioninae in Chinese waters.

In *Cyprinus* the pharyngeal bones are relatively well developed (Pl. II, fig. 7*d*, *e*). The length of each is only about two and a half times the width and the two limbs are of about equal length. They are thus massive bones compared with the other two types.

In all these types, the teeth are affixed to the medio-ventral aspect of each pharyngeal bone. In *Rutilus* they are close-set and uniserial, usually five in each row. Each has a cutting edge. The two posterior teeth (4 and 5) have recurved tips, forming hook-like processes directed backwards. These teeth, together with the third, are strongly compressed, but the first two teeth are stouter and more or less conical with pointed tips. Sometimes an additional tooth develops on the left side, a fact already noted by Hubbs and Hubbs (1944). In their discussion of 'bilateral asymmetry' in vertebrates they quote the roach (*R. rutilus*) as an example. Out of 104 specimens examined 79 had

asymmetrical teeth, the extra tooth being always on the left, never on the right. It should be noted that the extra tooth is only found in well-grown specimens, never in small ones. It therefore appears to be added at a later age.

The teeth in *Gobio* are biserial, each bone has a medial row with four or five teeth and a lateral row with two only. Each tooth is wedge-shaped and hooked as in the posterior teeth of *Rutilus*. The medial teeth are stronger than the lateral and, with the exception of the first which is small, of approximately equal size. The teeth of the lateral row lie opposite the third and fourth teeth of the medial row.

In *Cyprinus* the teeth are arranged triserially (2:1:1). In the mirror carp the rows are not quite so sharply defined as in the common carp in which sub-species there is also an extra tooth (3:1:1). The free ends of the teeth are rounded, somewhat pointed, but never hooked. All four teeth converge and collectively form a very strong, stud-like prominence, a feature regarded by Chu (1935) as of a highly specialized herbivorous type.

Small teeth may be found embedded in the mucous membrane of the pharynx in the vicinity of the pharyngeal bones. Chu regards these as accessory growing teeth destined to replace old teeth if these are lost.

A horny pad is developed in the dorsal wall of the pharynx below the occipital region of the skull opposite to the pharyngeal teeth. In *Rutilus* this is more or less ovoid in plan (Pl. I, fig. 1, H.P.) and double convex in transverse section (Pl. III, fig. 8) and fits loosely into a corresponding fossa in a special masticating process of the basioccipital. Its free surface is rugose and the pharyngeal teeth bite against it. This pad, together with the alternating disposition of the teeth, which are thus enabled to shear the food, completes an exceedingly efficient masticating apparatus.

The pad of *Cyprinus* is similar to that of *Rutilus* but even harder, but in *Gobio* the pad, trapezoidal in plan, is not nearly so well developed and is much softer (it can be easily pierced with a needle).

It follows from the above comparison that the entire pharyngeal dental apparatus is best developed in the herbivorous type (*Cyprinus*), facilitating the comminution of plant material, in the carnivorous *Gobio* the hooked teeth help to secure and tear the prey, while finally the omnivorous roach is strictly intermediate with both hooked and shearing teeth.

The gill-rakers are short in *Rutilus* and *Gobio*, but are longer in *Cyprinus*. Long gill-rakers characterize the majority of bottom-feeders which stir up the mud—a habit which the carp is known to have (Kyle, 1926). Thus, for example, they are well developed in the bottom-feeding *Mugil auratus* and *Upeneus barberinus* (Al-Hussaini, 1947*b*) and also in *Mulloides*, which is a bottom-feeder and shovels sand, and in *Scarus*, a coral feeder which grinds its food into a pasty mass (Al-Hussaini, 1946, 1945). The shortness of the gill-rakers in *Gobio* is therefore surprising, especially when one reads in Regan (1911), describing how *Gobio* secures its food, that it 'gropes and grubs' for it.

The Mechanism of Deglutition

The jaws are opened to admit food mainly by the action of two pairs of muscles, namely, the *geniohyoidei* (Text-fig. 1, GH.), a pair of stout muscles arising from the first and second branchiostegal rays and ceratohyals and passing forwards in close apposition to each other to be inserted on the inner surface of the dentary, and the *sternohyoidei* (Text-fig. 1, SH.) which arise somewhat laterally from the cleithra and pass forwards and towards the middle line to be inserted on the dorsal surface of the urohyal on either side of its dorsal crest. Thus by drawing the hyoid ventrally and caudally the sternohyoidei augment the action of the geniohyoidei.

The entry of prey or food into the mouth may be still further assisted by the action of the *levator arcus palatini* (Text-fig. 1, L.A.P.) which arises from the otic region of the skull and is inserted into the hyomandibular in *Gobio* and *Cyprinus* and into the hyomandibular and pterygoid in *Rutilus*. Thus by raising the hyomandibular (and pterygoid) the mouth cavity is enlarged and the food sucked into the mouth.

The closing of the mouth after the entry of the food is effected by the *adductor mandibulae*, a large, complex muscle lying immediately below the skin in the cheek region. In *Rutilus* two portions are distinguishable, the *maxillaris* (Text-fig. 1, MX.AD.) and the *mandibularis* (MD.AD.). The first of these arises from the preoperculum, passes below the eye, and is inserted by a strong tendon into the lateral surface of the dorsal portion of the maxilla. Its contraction lowers the maxilla slightly and thus holds the mouth firmly closed against the pull of the second portion, the mandibularis, on the lower jaw. The mandibularis portion arises from the hyomandibular, preoperculum, and quadrate, and runs immediately below the eye to its insertion by means of a strong tendon on the inner surface of the mandible. In *Gobio* both portions are further subdivided, the maxillaris into external and internal moieties, and the mandibularis into three more or less distinct parts. *Cyprinus* is intermediate in that the mandibularis portion is simple like that of *Rutilus* while the maxillaris is double and resembles that of *Gobio*.

In the living fish it may be observed that the roach is able to snap its mouth closed more quickly than either the gudgeon or the carp, an observation that may be correlated on the one hand with the single insertion for each portion of the adductor muscle in *Rutilus* against the multiple insertions in the other two species, and on the other with the feeding habits of the fish. Whereas *Rutilus* secures food which is moving freely through the water, *Gobio* and *Cyprinus* feed in a more leisurely manner by probing for sedentary food particles in the mud.

Once the food is securely within the mouth it may be assisted on its passage to the pharyngeal teeth by the narrowing of the mouth cavity. This is accomplished by two pairs of muscles. First, the *hyohyoideus* lies below the 'tongue' in the region of the geniohyoideus. Its action is to raise the 'tongue' and with it the floor of the mouth. In *Gobio* and *Cyprinus* this muscle comprises two distinct portions, with a greater extension ventral to the gullet

than in *Rutilus*. The second muscle constricting the buccal cavity is the *adductor arcus palatini*. It lies deep to the *levator arcus palatini* and arises from the parasphenoid and pro-otic and is inserted into the hyomandibula, pterygoid, and palatine, thus it draws these elements inwards and forwards. The area of insertion of this muscle is broader in *Gobio* and *Cyprinus* than in *Rutilus*.

As the food passes backwards into the pharynx the pharyngeal jaws are opened to admit it by the action of the *pharyngo-clavicularis*, a complex muscle with an 'externus' and an 'internus' portion. Both portions arise from the anterior surface of the cleithrum and pass in a generally dorso-medial direction. The 'externus' portion is inserted into the posterior surface of the anterior limb of the fifth branchial arch while the 'internus' portion is inserted partly on the ventral surface of the anterior extremity of the fourth branchial arch and partly in a similar position on the fifth arch. The action of this muscle is augmented by that of the trapezius (Text-fig. 1, TZ.) a strong muscle arising from the occipital and otic regions of the cranium and inserted into the tip of the posterior limb of the pharyngeal jaw. It is best developed in *Cyprinus*. The muscle, antagonistic to these two, which draws the teeth together and on to the surface of the horny pad is the *retractor arcus branchialis dorsalis* (Text-fig. 1, R.A.B.D.), a very powerful muscle, triangular in outline, arising from the masticatory process of the basi-occipital just lateral to the horny pad (cf. Pl. III, fig. 9), and inserted on the caudal surface of the posterior limb of the pharyngeal bone. Again it is best developed in *Cyprinus*, less so in *Rutilus*, and much less in *Gobio*.

Two other muscles are worthy of mention in connexion with feeding, namely, the *constrictor pharyngeus*, which passes laterally and somewhat caudally from the mid-ventral line of the pharyngeal wall, and the *intermandibularis* (Pl. I, fig. 2, IM.). This is a very small muscle (some 0·64 mm. wide in a roach 7 cm. long) passing between the medial surfaces of the dentaries; it is partially covered by the geniohyoidei. In the Cyprinids, where only small food particles are taken, this muscle remains small and only a slight movement is possible between the rami of the lower jaw (cf. also Takahasi, 1925), but in predatory types of mouth, e.g. *Pterois volitans*, the two dentaries may be widely separated during swallowing (Al-Hussaini, 1947*b*) when large prey is taken. Thus in this fish the intermandibularis is large, as it is in Catostomids (Takahasi, 1925; Edwards, 1926) and Siluroids (Takahasi, 1925).

Apart from the muscles described above, those operating the operculum and gill arches during respiratory movements by varying the hydrostatic pressure within the mouth and pharynx are bound to affect the passage of the food within.

As already mentioned small pieces of food are normally taken by a quick snap (less marked in *Gobio*), but where the prey is larger (e.g. earthworms) the fish first suck the food with great eagerness and then thrust it out again. The process is repeated several times till finally the fish, as it evades others, retires into a corner and indulges in what appears to be mastication. The

respiratory movements are accelerated and in particular the opercula are swung farther out than in normal respiration. The earthworm may be recovered from the gullet a short time later torn into pieces.

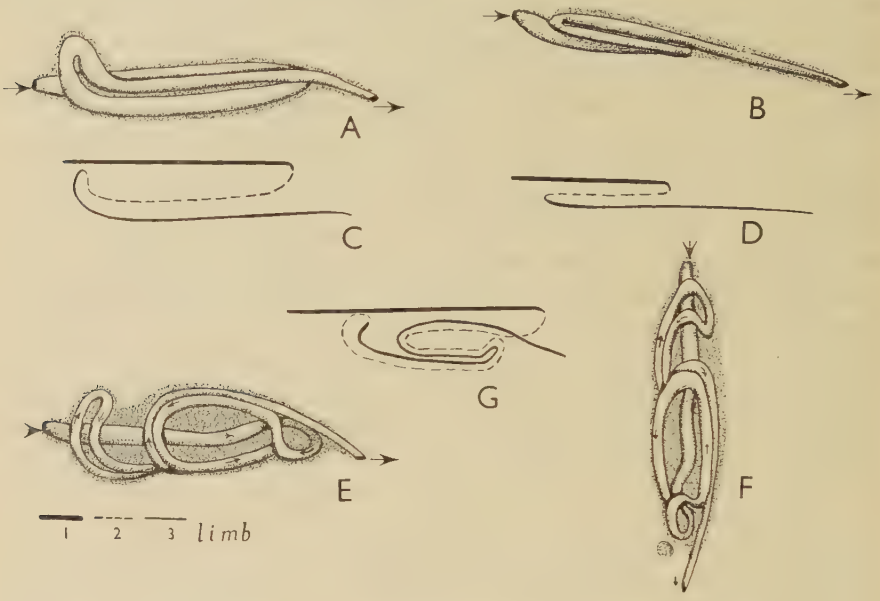
Thus in summary, the cycle of events occurring just before and during the capture of prey is as follows. The mandible is depressed and the premaxillae accordingly thrust forwards by the contraction of the genohyoideus and sterno-hyoideus, the motion of the mandible being transferred to the premaxillae by virtue of the ligamentous joint between them (Text-fig. 1, L.) the motion being limited by the length of the rostral ligament. As soon as the prey is seized and contained in the buccal cavity the two adductor mandibulae are simultaneously contracted, thus closing the mouth, the mouth cavity is narrowed by the contraction of the adductor arcus palatini and by the elevation of the mouth floor, thus pushing the food back towards the pharynx. The mouth is then opened again and the buccal cavity and the pharynx are dilated (the latter by the various muscles attached to the gill arches), and water is sucked in through the mouth. The prey is thus washed still farther backwards. In the next movement, that of closing the mouth, the pharyngeal floor is raised by the contraction of the constrictor pharyngeus, thus forcing the prey into the region of the teeth. Here, through the action of the retractor arcus branchialis dorsalis, trapezius, and pharyngo-clavicularis, the teeth can work alternately against each other, squeezing the prey between the two 'jaws' and against the horny pad, and finally pushing it backwards into the oesophagus. This 'masticating' action seems to be the more laborious part of the feeding process as observed in the living animal. It should be noted that a Cyprinid fish can hold its food and break it down by its teeth concurrently with the respiratory movements; in other words, deglutition and respiration do not interfere with each other. This is made possible by the position of the 'pharyngeal jaws' at the entrance to the gullet, that is posterior to the gills.

The Intestinal Tube

In all three examples the oesophagus, more or less cylindrical in form, occupies an oblique position in a cranio-caudal direction (Text-fig. 1; Pl. I, fig. 1, ES.), owing to the postero-ventral growth of the masticating process of the basi-occipital. It is remarkably short (4 mm. in a roach 175 mm. in length) and is delineated from the intestine by a constriction. The pneumatic duct (Text-fig. 1, P.D.), carrying a small dilatation at its extremity, opens into the dorsal aspect of the oesophagus.

The alimentary canal posterior to the oesophagus increases suddenly in diameter forming an intestinal swelling (Text-fig. 1, I.S.) as in all other Cyprinids so far described by various authors and in *Atherina* (Al-Hussaini, 1947*a*) and several labroid fishes (Al-Hussaini, 1947*b*). The swelling is a straight tube, gradually decreasing in diameter as it extends caudally, close to the dorsal body-wall, nearly as far as the posterior end of the air-bladder, from which point it curves sharply forwards again making one siphonal loop.

In *Rutilus* the distal limb of this loop, which reaches as far forwards as the transverse septum, is of smaller but constant diameter. It turns caudally once more and, coursing on the left of the siphonal loop, terminates at the anus. Sometimes the bend where the second and third limbs join one another has an upward tilt (Text-fig. 2A). The last portion of the third limb is tapering and corresponds to the rectum. Now it is true that in the cyprinids there is no ileorectal valve separating the intestine from the rectum as in most



TEXT-FIG. 2. The intestine as it appears *in situ* from the left side; A, *R. rutilus*; B, *G. gobio*; E, *Cyprinus carpio*; F, the intestine of *Cyprinus carpio* from the ventral aspect, C, D, and G diagrammatic representations of figs. A, B, and E respectively.

teleosts, but the fact that the last, tapering portion of the intestine has different histological features (cf. p. 134) is considered sufficient justification for designating it rectum. For a contrary view, reference should be made to Jacobshagen (1937), who considers that cyprinids, mormyrids, and some others do not have the mid-gut differentiated from the hind-gut.

In *Gobio* (Text-fig. 2B, D) the distal limb of the siphonal loop is short, the intestinal swelling extending only as far as the middle of the anterior lobe of the air-bladder before turning back. In *Cyprinus* (Text-fig. 2E, F, G) the intestine is longer and its looping more complex. The intestinal swelling, or first limb of the intestine, extends caudally throughout the whole length of the body-cavity, the intestine then curves sinistrally upwards and forwards to the middle of the body-cavity, and then backwards again in a sigmoid manner, becoming dextral once more as it approaches the posterior extremity of the body cavity. Here it doubles forwards again and courses anteriorly to the

septum transversum, taking an upward and sinistral course, then back once more with a hairpin loop, then forwards to the middle of the body-cavity and finally, with another sharp loop, it turns caudally and ultimately opens to the exterior at the anus. In the morphological sense the intestine of *Cyprinus* may still be regarded as formed of one and a half siphonal loops, as in the other two species, but the second and third limbs are strongly curved in the middle and shifted to the right (Text-fig. 2, compare G with C). This description was made from specimens of about 12 cm. in length, but according to Smallwood and Smallwood (1931) the intestine becomes much more coiled in large specimens of the common carp. It should be noted that the coils of the second and third limbs lie to the left of the first limb in all cases and that the proximal limb bears the swelling and is nearly straight in all three species, while the second and third limbs are shortest in the carnivorous *Gobio* and longest in the herbivorous *Cyprinus*.

The liver (hepatopancreas) grows round most of the intestine. The gall-bladder lies between the anterior lobe of the swim-bladder and the intestinal swelling, and its duct opens into the right side of the latter very slightly posterior to the oesophagus. The pancreatic duct accompanies the bile-duct, but it is difficult to find in gross anatomy.

Fatty tissue fills up the spaces between the three limbs of the intestine along its entire length and contains microscopic pancreatic alveoli embedded within it.

The blood-supply to the intestine is derived from a coeliaco-mesenteric artery which leaves the aorta just posterior to the pharyngeal dental apparatus.

The Mucosal Folds

In all three forms the mucous membrane along the inner edge of the upper jaw is produced into a crescent-shaped maxillary valve (Pl. I, fig. 1, MX.V.), papillated on its ventral surface. Contrary to the majority of teleosts an opposing mandibular valve is wanting, and thus the maxillary valve must operate against the floor of the mouth.

On the roof of the mouth there are several longitudinal folds which may be branched, or even swollen, and in the region opposite the 'tongue' they are papillated. In *Gobio* there are two distinct protuberances a little posterior to the maxillary valve. These will be referred to as 'palatine cushions'.

In all three species the pharynx is differentiated into two distinct regions, an anterior (Pl. I, fig. 1, A.PH.) and a posterior (P.PH.) proportioned to each other as 2:1. Functionally, the anterior pharynx containing the gill-slits is concerned with respiration, while the posterior pharynx containing the horny pad and pharyngeal teeth has an alimentary purpose. The mucosal folds increase in complexity as they run backwards (cf. Pl. I, fig. 1).

In *Cyprinus* the roof of the anterior pharynx is very 'fleshy' and exhibits an interesting response to mechanical stimulus—found to a less extent in the other two species. When pierced with a needle it swells considerably and then

gradually subsides. The reaction is shown even by a recently killed specimen from which histological samples were being taken.

The mucosal folds lining the intestine vary somewhat from one region to another. Various observations have been made concerning their pattern in sundry fishes by earlier workers, notably by Eggeling (1907) and Klust (1940).

In *Rutilus* there are some 10–12 longitudinal folds in the oesophagus which are continuous with the folds of the intestinal swelling. In the intestinal swelling the folds run in various directions, branch, and reunite. They are relatively broad, being about 0·33 mm. across when seen in surface view (Pl. I, fig. 1). The folds are particularly dense and crowded in the first centimetre of the swelling but become less so farther back. By the time the proximal part of the third limb of the intestine is reached, the mucosal folds are predominantly transverse in direction although they may still decussate. The distal five-sixths of the third limb exhibits a transverse zigzag pattern in the mucosal folds reminiscent of the arrangement of myomeres in the dogfish. The folds are also much thinner when seen in section (about four folds to the millimetre) and the mucosa has a brownish colour which is highly characteristic of this part of the intestine. About a centimetre before the anus is reached a faint line may be seen from the mucosal surface passing transversely round the intestine at the place where, in most fishes, the ileorectal valve occurs. The mucosal folds of the part of the intestine between this line and the anus (i.e. the rectum) assume a longitudinal direction, show only a few interconnexions, and become thicker once more.

In *Gobio* the mucosal folds resemble those of *Rutilus* except that those of the third limb of the intestine assume a longitudinal rather than zigzag orientation, and in the rectum the interconnexions between them become more numerous. In *Cyprinus* the folds of the third intestinal limb resemble those of *Gobio* but the condition in the rectum is more like *Rutilus*.

The four segments of the intestine can therefore be conveniently recognized by the following characteristics: the intestinal swelling, or first limb, has a greater diameter and the mucosal folds are complex, the second limb has a smaller and uniform diameter and less complex mucosal folds, while the third limb, similar in diameter to the second limb, is characterized by its brownish colour and narrow mucosal folds, and finally the rectum is tapering and its mucosal folds are longitudinal.

The Surface Area of the Intestinal Mucosa

The surface area of the intestinal mucosa is of great importance since it is this area which is concerned with the absorption of food. Warren (1939) adopted an excellent technique for measuring this in the dog which was modified by Wood (1944) and applied to cats and rats. The basic principle of the method is to inflate the intestine to a known and constant pressure (60 cm. of water for the dog) with saline and then fix it in this condition and finally cut micro-sections of it in transverse and longitudinal planes. These can be projected at a known magnification on to paper and the linear dimen-

sions of the serosal surface and of the mucosal surface in both directions can be measured by a rotometer. If now SC = serosal circumference, SL = serosal length, SA = serosal area, MC = mucosal circumference, ML = mucosal length, MA = mucosal area, then in general $\left(\frac{MC}{SC} \times \frac{ML}{SL}\right) > \frac{MA}{SA}$ for an irregular surface. However, as Warren shows, a serviceable approximation for the mucosal area (MA) can be obtained by the following formula, where EA = the estimated mucosal area. $\frac{EA}{SA} = \frac{MC}{SC} + \frac{ML}{SL} - 1$. ML, MC, SC, and SL are all measurable quantities, while SA is easy to calculate if the length of intestine under review is assumed to be a cylinder. This last assumption is not quite true for the fish gut since at one end the intestinal swelling and at the other the rectum are of inconstant diameter, and further, since it has been shown that the entire gut is absorptive (*vide infra*) the mere introduction and ligation of cannulae into the ends will produce an unavoidable error into the measurements of serosal and mucosal length. Nevertheless these errors are more or less constant for all three types examined and the data have therefore at least a comparable significance between them. Using Wood's modification, the precise procedure was as follows. The intestine was cut into two parts, viz. the first limb (swelling) in one part and the remainder of the intestine in the other. One end of each piece was attached to an end of the horizontal limb of a ⊥-shaped glass tube, the vertical limb of which was of such a length as to give the desired hydrostatic pressure to distend the intestine. The free ends of the two pieces of gut were then joined to the two limbs of a ⅄-shaped glass tube, the upright limb of which was connected to a wash-bottle to which a sphygmomanometer bulb was attached. On gently raising the pressure in the wash-bottle by means of the bulb the fluid contents of the bottle could be driven at a steady and constant pressure through the two portions of intestine simultaneously and up the vertical glass tube till it trickled out of the open end of this tube. The contents were first washed from the gut in this manner using the isotonic solution recommended by Young (1932) for fresh-water fishes followed by 10 per cent. formalin as a fixative. The data thus obtained are given below in Table 1.

Several interesting relationships may be deduced from this table. First the relative length of the gut (R.L.G., column 3) is nearly constant for each individual species, a feature already noted in other fish, but in *Rutilus* it is smaller than is usual for omnivorous species (1·3–4·3) while *Cyprinus* also has a smaller R.L.G. than the normal range for herbivorous fish (3·7–6·0); *Gobio*, on the other hand, lies within the normal range for carnivores (0·5–2·4) (Al-Hussaini, 1947*b*). The small R.L.G. of *Rutilus* and *Cyprinus* together with the simplicity of the gut (absence of stomach and pyloric caeca) may be peculiar to these species, but a considerably more extensive survey would be needed to settle this point.

The ratio between the estimated mucosal area and the serosal length (columns 6 and 9) is greater for the intestinal swelling than for the rest of the

TABLE 1. *Measurements of the Absolute Length of the Intestine, Mucosal Area, &c., in* Rutilus rutilus (R1–6), Gobio gobio (G7–12), *and* Cyprinus carpio (C13–15)

	1	2	3	4	5	6	7	8	9	10	11	12
Fish no.	*Length of fish less caudal fin in mm.* = FL	*Total length of intestine in mm.* — SL	SL/FL = R.L.G. (*relative length of gut*)	*Length of 1st limb of intestine in mm.* — SL_1	*Estimated mucosal area of first limb in cm.²* — EA_1	EA_1/SL_1	*Length of 2nd and 3rd limbs of intestine in mm.* = SL_2	*Estimated mucosal area of 2nd and 3rd limbs of intestine in cm.²* = EA_2	EA_2/SL_2	EA_1+EA_2 = *Total estimated mucosal area of intestine in cm.²* — EA	*Weight of fish in gm.* = WF	EA/WF = *mucosal coefficient* — QM
R1	95	100	1·05	20	8·36	4·18	80	23·85	3·18	32·21	14·0	2·39
R2	142	155	1·09	35	25·27	7·22	120	60·48	5·04	85·65	56·0	1·57
R3	155	165	1·06	45	42·08	9·35	120	74·16	6·18	116·24	75·5	1·53
R4	158	140	0·90	35	27·48	7·85	105	71·19	6·78	98·67	66·0	1·50
R5	185	183	1·00	48	54·91	11·44	135	102·51	7·63	157·42	104·0	1·51
R6	185	185	1·00	46	51·38	11·17	139	114·85	8·27	166·23	105·5	1·58
G7	101	69	0·68	21	11·13	5·30	48	15·55	3·24	26·68	11·0	2·42
G8	103	74	0·72	25	13·75	5·50	49	17·98	3·67	31·78	13·1	2·41
G9	104	75	0·72	25	15·73	6·29	50	18·75	3·75	34·48	16·0	2·15
G10	116	88	0·76	30	22·53	7·51	58	18·44	3·18	40·97	19·2	2·13
G11	119	101	0·85	32	21·54	6·73	69	25·46	3·69	47·00	22·0	2·14
G12	120	102	0·85	32	20·54	6·42	70	28·91	4·13	49·45	23·2	2·13
C13	75	140	1·87	20	7·66	3·83	120	21·48	1·79	29·14	11·5	2·53
C14	76	140	1·84	20	9·62	4·81	120	24·96	2·08	34·58	13·5	2·56
C15	98	180	1·84	30	16·92	5·64	150	33·00	2·20	49·92	20·2	2·50

intestine, a feature obviously to be correlated with the complexity of the mucosal folds. Further, although the estimated mucosal area is *absolutely* greater in the larger compared with the smaller fish (cf. columns 1, 10, and 11) because the mucosal foldings grow with the fish, nevertheless it is *relatively* smaller (cf. R1 with R5 and R6). In this connexion it must be remembered that the animal in its growing stage needs a relatively greater quantity of food and hence has a greater absorptive area per unit of body-weight. This ratio between the mucosal (absorptive) area and the body-weight (mass of tissue requiring food), which I have called the *mucosal coefficient*, is expressed numerically in column 12. So far as mammals are concerned Wood (1944), working on cats and dogs of practically equal weight, found by measurement that the ratio is constant in both mammals, while Cori (1925), using growing rats of varying weight, deduced a constant ratio from the quantities of sugar absorbed.

Referring again to the Cyprinid fish, it may be seen that in *Gobio* the mucosal coefficient becomes nearly constant by the time the fish has attained a length of 110 mm. and a weight of about 15 gm. Again, if the mucosal coefficients of the three fish are to be compared and related to their varied diets it should be done with fish of approximately the same weight. This has been done in Table 2.

TABLE 2. *Relative Length of Gut and Mucosal Coefficient of Three Species of Fish of approximately Equal Weights*

Species	*No. in Table 1*	*Intestinal length in mm.* = SL	*Body-weight in gm.*	*Estimated mucosal area in cm.²* = EA	*Mucosal coefficient*	R.L.G.
Gobio gobio . .	G8	74	13·1	31·78	2·41	0·72
Rutilus rutilus . .	R1	100	14·0	33·52	2·39	1·05
Cyprinus carpio . .	C14	140	13·5	34·58	2·56	1·84

An examination of the table shows that *Cyprinus* has the highest R.L.G. and *Gobio* the lowest, and although the mucosal area follows the same sequence the difference is very much less marked. Thus one is led to the important deduction that the shortness of the intestine may be compensated by an increase in the complexity of the mucosal foldings. It should, nevertheless, be borne in mind that the longer intestine, although having a mucosal coefficient very little greater than the short one, probably has the advantage of retaining the food for a longer period and may still be regarded as an adaptation to herbivorous diet provided that the comparison is made between closely related species living under fairly similar conditions.

The Histology of the Alimentary Tract

For this study serial sections were made from specimens measuring 65–100 mm. long. The material was fixed and decalcified simultaneously in

Boling's modification of Bouin's picro-formol (Cowdry, 1943, p. 188) and stained in Delafield's haematoxylin-eosin, certain details being subsequently verified by using Mallory's triple connective-tissue stain, Mayer's muci-carmine, or Giemsa's method for paraffin sections. Individual cell types were studied by the maceration method of Goodrich (1942). In addition to these methods the oesophagus and intestine were removed from specimens of different sizes and fixed in Bouin, Maximow, or Susa and stained with Heidenhain's iron-alum haematoxylin-eosin.

The Buccal Cavity

The buccal cavity of *Rutilus* is lined by a stratified epithelium containing taste-buds and mucus-secreting cells and resting on a basement membrane supported by a stratum compactum (Pl. I, fig. 2). The sub-epithelial connective tissue is differentiated into a tunica propria in juxtaposition to the epithelium and a deeper, looser tissue known simply as the submucosa, a condition similar to that found in all fishes studied earlier (Al-Hussaini, 1945–7).

At the entrance to the mouth cavity the epithelium is thick and the outermost layers are formed of strongly cornified polyhedral cells. In these respects it resembles the epithelium of the outer skin but differs from it in that both mucus-secreting cells and taste-buds are absent; both of these structures occur in the skin. Further, the 'Kolbenzellen', characteristic of the skin of certain teleost families (Uhlich, 1937), are absent. Such cells have been described from the buccal epithelium of *Calamoichthys* (Purser, 1926). They have a central nucleus embedded in a clear cytoplasmic fluid which does not show the staining reactions characteristic of mucus.

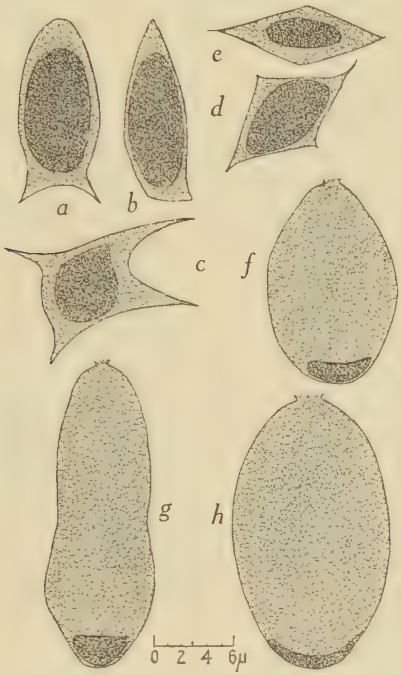
A very short distance within the mouth opening taste-buds begin to appear in the sections and rapidly become numerous as one passes backwards; they are particularly numerous on the ventral surface of the maxillary valve.

According to Herrick's (1903–4) view of the direct correlation between the density of taste-buds and the delicacy of the sense of taste in fishes, *Rutilus* should have a well-developed sense of taste in both the skin and the mouth. In *Rutilus* each taste-bud is a flask-shaped structure measuring about 100μ by 20μ and in general structure similar to those already described in *Mulloides* (Al-Hussaini, 1946).

By the time the middle of the maxillary valve is reached taste-buds have become the predominant feature of the buccal epithelium and are found all over the mouth cavity except on the dorsal surface of the maxillary valve. On the other hand, mucus-secreting cells, which have already made their appearance at this level, are plentiful just where taste-buds are absent or sparse, namely, on the dorsal surface of the maxillary valve and the roof of the mouth. Towards the posterior end of the valve taste-buds and mucus-secreting cells are nearly equally distributed on both the roof and floor of the mouth. At the sides of the mouth, which in transverse sections appear as two recesses, both elements are scarce.

The mucus-secreting cells are mostly of the pyriform type (cf. Al-Hussaini, 1945, p. 368, for terminology of mucus-secreting cells) and are concentrated along the crypts of the buccal epithelium rather than on the folds. Although assuming no particular pattern they become more numerous as one passes caudally towards the pharynx while the taste-buds show a reverse tendency (Text-fig. 4).

A possible explanation of this differential distribution is that the mucus secreted by numerous cells on the opposing surfaces of the dorsal aspect of



TEXT-FIG. 3. Constituent cells of the stratified epithelium of the bucco-pharynx of *R. rutilus* obtained by Goodrich's maceration method: (*a*) from the stratum germinativum; (*b*) from the second layer; (*c*, *d*) from intermediate layers; (*e*) from the superficial layer; (*f*) a pyriform and (*g*, *h*) saccular mucus-secreting cells.

the maxillary valve and the roof of the mouth prevents their adherence and facilitates the movement of the valve during respiration. They are otherwise absent from the extreme anterior part of the mouth since lubrication of the food is not necessary until after the food is in the mouth, and swallowing has commenced. The taste-buds, on the other hand, are concentrated very early so that the food may be immediately sampled.

The mouth floor, or 'tongue' region, shows no special histological features which differentiate it from other areas of the mouth.

The cells in the basal layer (Text-fig. 3*a*) are formative and from them the other cell types are derived. The cells of the intermediate layers are frequently polyhedral and have their angles drawn out into short processes which, by

interlocking with similar processes from neighbouring cells, presumably give a firmer and tougher structure to the entire epithelium. Within the layer of cells immediately adjoining the basal layer certain spheroidal cells may be found, each with a short process housing the nucleus. Similar cells may be found in the succeeding layers as the surface is approached, but they tend to get larger and the nucleus becomes compressed at the base rather than contained in a process (*f*, *g*, *h*). It would thus appear that the pro-mucus-secreting cells are differentiated along with the stratified cells from the basal layer, and that mucus secretion is commenced very early in the course of their differentiation, the amount of secretion being gradually increased as the cells approach the surface.

The basement membrane, stratum compactum, tunica propria, and submucosa are similar in general structure to those described in the earlier papers. In the floor of the mouth, in the region of the 'tongue', the submucosa contains large adipose cells which persist back to the heart region.

In both *Gobio* and *Cyprinus* the barbs on the lips are very richly supplied with taste-buds, but, in addition to these, taste-buds are much more abundant both on the lips (Pl. I, fig. 3) and in the buccal epithelium than in the roach.

The order of the three fish on the basis of the abundance of taste-buds is *Cyprinus*, *Gobio*, *Rutilus*. It is surprising to read in Curry's account (1939) that taste-buds are not numerous in the floor of the mouth of the common carp and that they 'are more numerous on the flap than in any part of the buccal cavity'. By 'flap' she presumably means the maxillary valve. In contrast to this the mirror carp possesses very numerous taste-buds over the entire lining of the buccal cavity *except* on the dorsal surface of the maxillary valve (Pl. I, fig. 2). In fact I have not yet examined a fish with more abundant taste-buds than the mirror carp, and, further, they are of larger size than those of either *Gobio* or *Rutilus*.

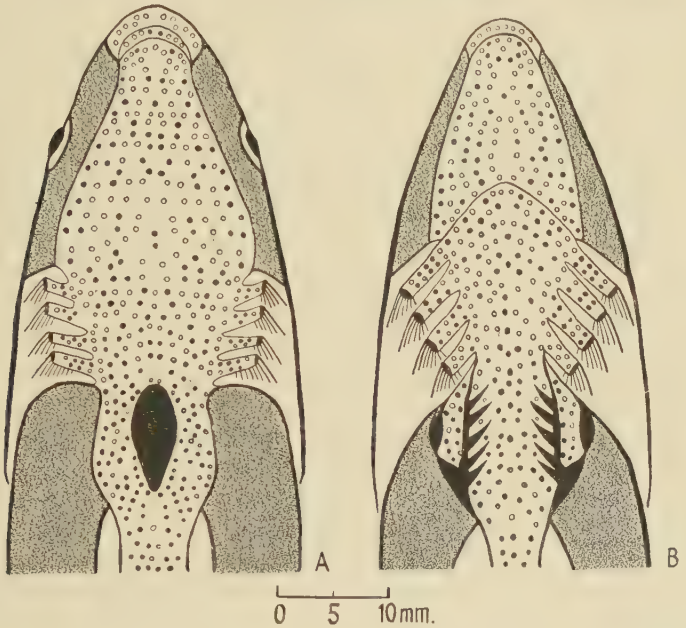
In *Gobio* the two convex 'palatine cushions' before mentioned (p. 119) are richly supplied with taste-buds and pyriform mucus-secreting cells, the latter being specially concentrated along the sides of the crypt which separates the two cushions. The sub-epithelial connective tissue is richly vascular, and contains numerous collagen fibres and nerves. An attempt was made to demonstrate the action of these cushions experimentally by first anaesthetizing a fish and then presenting it first with a piece of gravel, and then with food (meat) of similar size held close to the mouth. Every time without exception the particles would be sucked in with the respiratory current and on touching the cushions the gravel would be immediately rejected, but the food particles would be retained, at least for some time. It is thus clear that the cushions act as food selectors and, at the same time, act as an efficient barrier to the passage of all other material entering the mouth, allowing it to be immediately rejected. The significance of such a mechanism is self-evident when one recalls the bottom-feeding, mud-grubbing habits of the fish. The highly specialized gustatory sense of both *Cyprinus* and *Gobio*, in which they also

resemble *Mulloides auriflamma* (Al-Hussaini, 1946), is clearly an adaptation to the bottom-feeding habit.

The Pharynx

The Anterior Pharynx

In all three fish the mouth merges gradually, with no abrupt change, into the pharynx. The mucus-secreting cells become gradually more numerous and tend to become concentrated along the sides of the crypts so that, in



TEXT-FIG. 4. Diagrammatic representation of the distribution of taste-buds and mucus-secreting cells in the fore-gut of *R. rutilus*. A, the roof; B, the floor of the mouth, pharynx, and oesophagus. Taste-buds open circles (o), mucus-secreting cells solid (●).

transverse section, they have the appearance of multicellular glands (Pl. II, fig. 5). In some places they become huddled together and stratified, and encroach upon the undifferentiated epithelial cells which, although multi-layered high up on the sides and on the crests of the folds where mucus-secreting cells are few, are reduced to a single layer at the bases of the crypts. This arrangement resembles that found in other fishes I have previously investigated and described.

Taste-buds remain numerous, especially in *Gobio* and *Cyprinus*; in the lateral portions of the anterior pharynx of the latter fish especially they form an almost unbroken sequence (Pl. I, fig. 4), while on its roof there is the palatal organ of Valatour, with its acknowledged gustatory function (Herrick, 1904).

The foregoing account concerning taste-buds and mucus-secreting cells conforms closely to the generalization which I drew concerning the pharynx in teleosts (Al-Hussaini, 1946), namely, that it is a region concerned with gustation as well as mucus production, irrespective of the kind of diet of the fish, and that the relative extent of these two functions is slightly altered when the food contains a great amount of silt, as, for example, in *Scarus* and *Mulloides*. An additional feature is a high concentration of both taste-buds and mucus-secreting cells on the gill-rakers in the Cyprinids (Pl. II, fig. 6). Presumably the mucus serves to trap food particles detected by the taste-buds and so prevent their ejection with the respiratory current, but an attempt to demonstrate this experimentally led to indefinite results since direct observation of this region of the pharynx could only be made at the expense of normal respiratory movement.

Concerning the sub-epithelial structures of the anterior pharynx in general the following noteworthy points may be mentioned. The stratum compactum is thinner than in the mouth, in fact in some places it is difficult to detect. In the tunica propria there are numerous striated muscle fibres (Pl. I, fig. 4). In general these are arranged singly and transversely but they may run in various directions and may be assembled in groups. A striking feature of these muscle-fibres is that in several places they are closely associated with the stratum compactum, while they also invade the cores which support the taste-buds. They presumably serve to mobilize these structures as well as the general mucosa of the pharynx during the processes of mastication and swallowing. The large adipose cells already noted in the submucosa of the floor of the mouth extend also into the pharynx and are found in addition in the roof but not at the sides of this cavity.

The submucosa of the pharyngeal roof is much thicker in *Cyprinus* than in either *Gobio* or *Rutilus*, thus accounting for its more 'fleshy' appearance, while its nerve-supply is very rich—a feature obviously to be correlated with the profusion of taste-buds found in this species.

The Posterior Pharynx

The mucosal folds of this region are high; they branch and anastomose freely. Mucus-secreting cells abound (Text-fig. 5*c*) and attain their maximal development in this region, being of the saccular type (Text-fig. 3*g*, *h*).

Taste-buds are still numerous, although in *Rutilus*, but not in *Gobio* or *Cyprinus*, they become much less abundant posteriorly.

The horny pad, characteristic of the roof of the posterior pharynx, consists of an especially thick stratified epithelium (Pl. III, fig. 8). Of the three easily recognizable zones the basal (B.Z.) consists of a single layer of cells following the sinuosities of the tunica propria underlying it. The middle zone (M.Z.) comprises large polygonal cells, the cytoplasm of which stains deep red with eosin and crimson with Mallory's triple stain, while the nuclei remain pale and show nucleoli. The cell membranes are much thickened, and fine cytoplasmic bridges connecting neighbouring cells together are clearly visible.

The number of layers of cells forming the superficial zone (s.z.) is about equal to those of the middle zone in *Rutilus* but is smaller in *Gobio* and larger in *Cyprinus*. Although the individual cells of the superficial zone also have thick membranes they show a highly characteristic and very irregular outline as if crumpled and distorted. They take a bright-red colour with eosin and stain orange with Mallory: thus the superficial zone is sharply contrasted to the one below it. The nuclei are retained but in a very degenerate condition, although Curry (1939) described and figured similar cells in the common carp as 'enucleated'. The superficial cells are shed from the surface either singly or in groups. The superficial zone does not occur on the anterior or posterior extremities of the pad, which exhibits here a somewhat attenuated form. The staining reactions of this zone are those characteristic of horn, and the hardness of the pad is in direct proportion to its thickness, being greatest in the herbivorous and least in the carnivorous example.

Numerous coarsely striated muscle-fibres are found at the base of the pad, which presumably would be capable of effecting some adjustment of its position. The stratum compactum is very thin in the posterior pharynx and is composed of only a few compact collagen fibres.

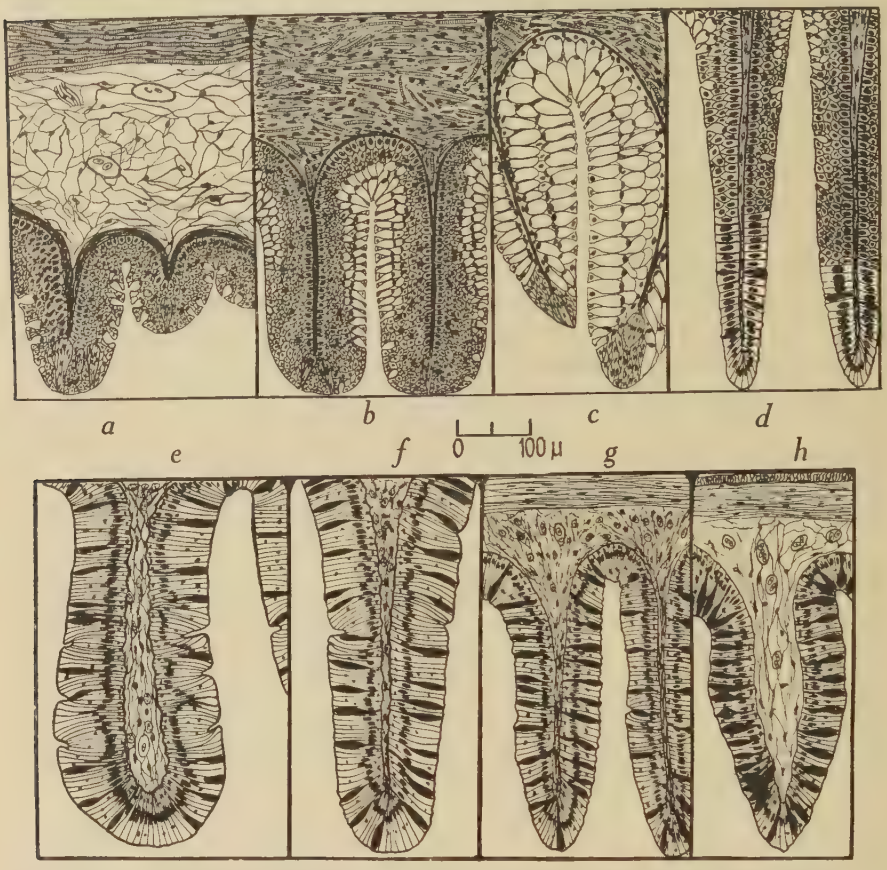
The histology of pharyngeal teeth has already been described in *Mulloides* (Al-Hussaini, 1946) and the present fishes exhibit similar features except that their teeth are firmly fixed to the underlying pharyngeal bones.

The Oesophagus

The oesophagus is customarily defined in higher vertebrates as the transitional region between the pharynx where the glottis opens and the stomach, so on this basis it must be regarded in the Cyprinids as the region of the gut extending from the horny pad and pharyngeal teeth to the point where the intestinal epithelium begins, or rather to the constriction (pyloric sphincter) at the commencement of the intestine (Pl. III, fig. 9). Only the ventral and lateral aspects of the oesophagus are covered by serosa, immediately beneath which is a dense layer of striated muscle. This single muscle layer, although of a general circular character, is not precisely transverse to the longitudinal axis of the oesophagus which is itself oblique. At the junction between oesophagus and intestine the oesophagus undergoes a pronounced thickening and forms a pyloric sphincter (M.C.). A pyloric sphincter has been recorded in the goldfish by McVay and Kaan (1940), but no reference was made to it by either Rogick (1931) in the minnow or Curry (1939) in the common carp. The submucous connective tissue is very compact and contains many coarsely striated muscle-fibres which run haphazardly in various directions and which extend up the very base of the mucosal epithelium, being separated from it only by a very thin stratum compactum consisting of a very few collagen fibres.

The mucosal folds of the anterior end of the oesophagus resemble, in general, those of the posterior pharynx; their branching system, giving rise to primary and secondary folds, has already been described for the common carp by Edinger (1877) and Oppel (1896). Taste-buds are still plentiful in *Gobio*

and *Cyprinus* but are very scarce in *Rutilus*, while mucus-secreting cells of the saccular type are present in all three. At the caudal end of the oesophagus the mucosal folds become very much deeper and the stratified squamous epithelium characteristic of the crypts and bases of the folds changes to a



TEXT-FIG. 5. Schematized drawings of individual mucosal folds from various parts of the alimentary canal of *R. rutilus*. Only the goblet cells, among the various types of mucus-secreting cells, are shown in black. (*a*) buccal cavity; (*b*) anterior pharynx; (*c*) posterior pharynx, lateral wall; (*d*) oesophagus; (*e*) intestinal swelling; (*f*) second limb of intestine; (*g*) third limb of intestine; (*h*) rectum.

columnar epithelium as the crest of the fold is approached (Text-fig. 5*d*). This columnar epithelium contains mucus-secreting cells of the goblet type only and no taste-buds.

The arrangement just described is the reverse of that found in the common carp by Curry (1939), while in the minnow Rogick (1931) described a mixture of epithelial types in the mucosal folds of the oesophagus.

From the foregoing study and from knowledge gained from three fish studied earlier (*Scarus*, *Mulloides*, and *Atherina*, Al-Hussaini, 1945, 1946,

1947*a*) it is possible to reach two important conclusions concerning the morphology of the oesophagus in teleosts. Firstly, the mucosa changes gradually from a stratified epithelium with saccular mucus-secreting cells to a columnar epithelium with mucus-secreting cells of the goblet type. Secondly, its muscular coat consists of a dense layer of striated fibres circular in direction. In *Atherina* alone a layer of longitudinal fibres occurs internal to the circular layer, and in all the examples studied the submucosa, and even the tunica propria, always contains scattered muscle-fibres. The pyloric sphincter is formed by the thickening of the circular layer.

Thus the oesophagus is distinguished from the pharynx on the one hand by its first mixed and then purely columnar epithelium, and from the intestine on the other by the type and arrangement of its musculature (in fishes with a stomach the multicellular 'cardiac' glands may serve as a posterior diagnostic boundary). It is hoped that this account may help in clearing away the vague definitions so much in vogue. As thus defined the oesophagus of the Cyprinids here studied shows no adaptation to feeding habits apart from the fact that taste-buds are more extensive in the anterior region in *Gobio* and *Cyprinus* than in *Rutilus*.

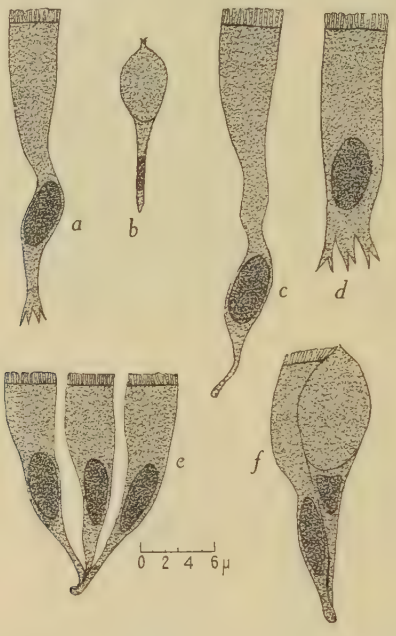
Immediately posterior to the horny pad the pneumatic duct opens into a short dorsal diverticulum formed by two major folds of the oesophagus, at which point the mucosal cells of the oesophageal folds become columnar. The duct, near the opening, is ensheathed by a thick layer of fibrous tissue; to the outside of this is a dense layer of longitudinal striated muscle-fibres which is in turn surrounded by a similar layer of circular fibres. Presumably these fibres regulate the opening and closing of the aperture of the duct.

The Intestine

The intestine is lined by a simple columnar epithelium comprising only two principal types of cells but containing also two or three other subsidiary cell-types which will be dealt with in detail below. The more common of the two principal types is that usually described, even by recent authors (e.g. Maximow and Bloom, 1945, and Clark, 1945), as 'columnar' or 'cylindrical' on account of their appearance in normal micro-sections. As will be shown later, the shape thus seen refers to a part of the cell only; the other part, not easily seen in sections, is variable according to the position of the cell in the mucosal fold. Following the lead recently given by Baker (1944) the term 'absorptive cells' is used in the present paper, thus putting emphasis on their function rather than their shape.

The true shape of these cells is revealed by the maceration method of Goodrich (1942). It will be seen (Text-fig. 6) that the majority are not straight but, in addition to a general tapering towards the base, each cell is bent at least once. These bends do not all occur at the same level but interlock with similar bends in neighbouring cells and this, presumably, serves to strengthen the epithelium as a whole. The nuclei are variously disposed within the basal portions of the cells; their presence always involves a bulging of the cell

outlines, which by interdigitating with the swollen nuclear portions of neighbouring cells further serves to strengthen the epithelial sheet. The basal terminations of the epithelial cells, which may be knob-like (*c*, *f*) or finger-like (*a*, *d*), serve to anchor the cells to the subjacent tissues. Sometimes several cells may be anchored together by this means (*e*). The free border, at least in the intact epithelium, seems to be continuous from one cell to another.



Text-fig. 6. Drawings of isolated cells from the intestinal epithelium of *R. rutilus*. (*a*), (*c*), (*d*), and (*e*), absorptive cells; (*b*) goblet cell; (*f*) absorptive and goblet cells in apposition.

Only two previous attempts to study the intestinal cells of fishes by a maceration method appear to have been made. The first, by Edinger (1877), working with the common carp, reveals simply an inverted conical structure ending in a filamentous process, and shows neither the perinuclear swelling nor the terminal nodules or processes. The second attempt was by Stirling (1884), using the herring as material. His figures of the cells between the oesophagus and the cardia of the stomach resemble Edinger's except that the filamentous basal process is shorter.

Macklin and Macklin (1932) put forward the idea that the spaces between the filamentous processes of the absorptive cells are not separate intercellular spaces but one continuous space, filled with fluid which freely communicates with the tissue fluid in the cavities of the sub-epithelial core, either by diffusion through the permeable basement membrane or possibly by actual minute perforations. Thus what may be termed the 'excretory

area', or the surface area across which substances may diffuse from the absorptive cells to the body fluids, is enormously increased by this tapering of the basal portions of the epithelial cells. In *Gobio* the cells are shorter than in *Rutilus* and *Cyprinus* and hence the 'excretory area' is accordingly smaller.

The second of the two principal cell-types found in the intestinal epithelium is the mucus-secreting cell. These are typical goblet cells (Text-fig. 6*b*, *f*) which attain their maximum concentration in the rectal epithelium (Text-fig. 5*e–h*)—a fact which, surprisingly enough, has hitherto escaped the attention of investigators. This high concentration of goblet cells in the rectal mucosa obviously facilitates defaecation.

Other cellular structures which may be observed within the intestinal epithelium include small lymphocytes which, although commonest within the fluid-filled intercellular space around the bases of the absorptive cells, may migrate between them to within a short distance of the free border. A second type consists of granular cells (absent from *Gobio*). These are particularly abundant in the submucosa and, although they may invade the basal part of the epithelial layer, they do not migrate so near to the free border as do the lymphocytes. (This is in marked contrast to the condition observed in the three fish studied earlier, Al-Hussaini, 1945, 1946, 1947*a*.) They may also be found in the connective tissue between the two muscle layers and beneath the serosa and will be discussed in more detail in Part II of this paper. Finally, a third type of cell, of infrequent occurrence, comprises cells, pear-shaped in outline, which reach the free border and are recognizable by their cytoplasmic inclusions and spheroidal nucleus. Their precise significance is obscure and they will be dealt with in more detail in a subsequent paper.

Regarding the sub-epithelial tissues, the stratum compactum, better developed in *Gobio* than in *Rutilus* or *Cyprinus*, lies in close proximity to the epithelium and not in the middle of the submucosa as in the Salmonidae (Oppel, 1896; Greene, 1912). If the view that the stratum compactum acts as a girdle, checking undue distension of the intestine (Baecker, 1940), be accepted, then the intestines of *Rutilus* and *Cyprinus* will be more distensible than that of the carnivorous *Gobio* since their strata compacta are weaker, a feature that may be correlated with the more bulky nature of their food.

The muscular coat consists of an inner circular and an outer longitudinal layer. A transition from striated to non-striated muscle occurs in the circular layer immediately caudal to the pyloric sphincter and in a roach 7 cm. long is complete by 2·2 mm. beyond the choledochal duct. The unstriated fibres first appear internal to the striated fibres, and the transition is accomplished gradually, so that, in progressively caudal transverse sections, the striated layer becomes gradually thinner and the non-striated wider.

Using the common carp as an example of teleosts, Li (1937) described a thin layer of longitudinal fibres next to the submucosa. Using the same technique on the Cyprinids now being considered it has not proved possible to demonstrate a continuous sheet of longitudinal fibres in this position, although in a few places isolated patches of such fibres were seen. A few points remain to

be dealt with concerning the intestine. The choledochal duct unites with the pancreatic duct and then immediately opens into the intestinal swelling, not, as described by McVay and Kaan (1940) in the goldfish, on the summit of a papilla, but simply between two mucosal folds, their epithelium becoming continuous with that lining the ducts.

In the rectum the submucosa is richly vascular, and since the mucosal folds are shallower and broader than in the intestine the sub-epithelial cores are more extensive. The anal outlet is lined by a stratified epithelium containing numerous pyriform mucus-secreting cells like those of the skin, from which, indeed, it is distinguished only by the absence of 'Kolbenzellen' characteristic of the latter. The circular muscle thickens to form an anal sphincter. In all the three cyprinids the luminal surface of the epithelium of the intestinal swelling is commonly indented (Text-fig. 5*e*) so that the surface is lowered forming a pit-like depression. A central cell extends in a straight line from the pit to the submucosa, but the cells around it are curved so that they form collectively a sort of 'nest'. These cells have a more or less uniform breadth, their nuclei are not compressed, and their cytoplasm stains but feebly; the cells around the 'nest', however, are much compressed, their nuclei elongated, and their cytoplasm stains more deeply. These 'nests' of cells also occur in the second limb of the intestine, though less commonly, while in the third limb of the intestine and rectum they are rare. Their significance is unknown; they cannot be homologous with primordial gastric glands because they are post-pyloric in position.

Discussion

Biologists practically agree that the roach is typically omnivorous, the gudgeon predominantly carnivorous, and the mirror carp mainly herbivorous; the small quantity of vegetable material taken in by the gudgeon and of animal food eaten by the mirror carp merely serving to 'balance' the diet (cf. Kyle, 1926). The following discussion is based on this assumption.

None of the three types possesses teeth on either jaws or palate, but all have well-developed pharyngeal teeth firmly affixed to the modified fifth gill arch which oppose a horny pad carried on a special masticatory process of the basi-occipital. Mastication is thus effected in the pharyngeal region, as it is in scarids and labrids which crush respectively coral and molluscan shells, and hence does not obviously interfere with respiration since it takes place posterior to the respiratory part of the gill apparatus.

The form of the teeth also shows a correlation with the type of diet. Thus the carnivorous *Gobio* has biserial hooked teeth, the omnivorous *Rutilus* has uniserial teeth, some of which are hooked, while the herbivorous *Cyprinus* has three rows of teeth converging towards each other forming a stud-like 'molariform' prominence. Both the relative overall size of the pharyngeal jaws as well as of the muscles which actuate them are best developed in *Cyprinus* and least in *Gobio*, while the hardness of the horny pad is also in direct relationship to the amount of plant food ingested.

Of the sixty-odd species of teleosts previously examined and described (Al-Hussaini, 1947*b*), most of the herbivorous species have serrated, 'incisiform' oral teeth and very weak pharyngeal ones. They are thus capable of nibbling the plants by means of their front teeth and the food enters the mouth in a ready-macerated form. In the grey mullets and also, according to Wier and Churchill (1945), in the gizzard shad (*Dorosoma cepedianum*), the pylorus forms a 'gizzard' which triturates diatoms and the like. Fishes living on plant food or hard food have thus acquired one mechanism or another which enables them to mince their food in preparation for digestion; therefore the correlation of the morphological features of the alimentary tract with the feeding habits is complicated by the genetical factor. For example, the carnivorous *Gobio* is more closely comparable to the herbivorous *Cyprinus* than the latter is to a herbivorous acanthurid like the doctor fish, *Acanthuras sohal*, which possesses oral teeth, a stomach, and pyloric caeca. When *Gobio* and *Cyprinus* are compared with each other, however, several significant points of difference become apparent, as, for example, the stronger 'molariform' teeth in *Cyprinus* and the longer intestine. It is consequently not always possible to draw hard-and-fast lines between the several groups of feeders based on certain characteristic features, because fishes, during their progressive evolution, have become variously adapted to the conditions surrounding them, diverging gradually from each other in the process. An excellent example is afforded by the three herbivorous species *Acanthurus sohal*, *Cyprinus carpio*, and *Mugil auratus* belonging to three distinct families. The plant food on which these fishes graze is comminuted by the serrated 'incisiform' oral teeth in the first, by the pharyngeal teeth in the second, and by the gizzard-like pylorus in the third species—three different structures, but all, in fact, fulfilling a similar function. On the other hand, *Gobio* can be compared with *Mulloides* and *Cyprinus* with *Mugil*. The first two are carnivores and the last two are herbivores, but all four are bottom-feeders, stirring up the mud and seeking their food from it, and all lose their oral teeth.

Although the cyprinid gut is simple and its relative length (cf. p. 121) is smaller in *Rutilus* and *Cyprinus* than for the average omnivorous or herbivorous fish, yet estimations of the mucosal area (made here for the first time) show that this is not small relative to that of mammals, and that variations in the relative length of the gut may well be compensated by variations in the mucosal area (cf. p. 123 for a fuller discussion of these matters).

Turning now to histological features we find that the abundance of taste-buds is rather to be correlated with the way in which the fish secures its food than with its nature. Thus when a fish has to select its food from mud (*Gobio* and *Cyprinus*) or sand (*Mulloides*), or to single out living coral from amongst inorganic material (*Scarus*), an efficient gustatory sense is imperative, especially around the entrance to the mouth cavity, and does not depend on the nature of the nutritional substances: and taste-buds are much more numerous in the species named than in the free-feeding, omnivorous *Rutilus* or the

plankton-feeder *Atherina*. Nevertheless, taste-buds are quite common structures within the mouth in *Rutilus*.

The histology of the teleostean intestine is one of the simplest among vertebrates. In the Cyprinids under consideration the intestinal epithelium shows no special features beyond the formation of the special cellular 'nests' referred to above (p. 134); indeed, multicellular glands are characteristically absent from the teleostean intestine except in some of the Gadidae (Jacobshagen, 1937). A spiral value is present in *Chirocentrus*, while vestiges of it remain in some of the Salmonidae and in *Gymnarchus* (Goodrich, 1909); the typhlosole-like structure found in *Scarus* may well be peculiar to this genus (Al-Hussaini, 1947*b*). A rectal gland is never present in teleosts but pyloric caeca frequently are (cf. Al-Hussaini, 1946 and 1947, for a fuller discussion of these structures).

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Summary

1. The mouth is larger and more protrusible in *Gobio* and *Cyprinus* than in *Rutilus*, features which may be associated with the mud-grubbing, bottom-feeding habits of the two former fish, while the arrangement of the adductor muscles in *Rutilus* enable it to close its mouth more quickly than they and hence it can rapidly snap up its free-moving prey.

2. The selection of food is largely by taste in *Gobio* and *Cyprinus*, and hence both are richly supplied with taste-buds from lips to oesophagus, special concentrations being present on barbs around the mouth. *Rutilus*, on the other hand, augments taste by sight. Taste-buds are accordingly more restricted in distribution and less abundant where they do occur. They are fewer on the lips; there are no barbs.

3. The relative development of the pharyngeal masticatory apparatus (horny pad, pharyngeal teeth) bears a direct relationship to the amount of plant food in the diet, i.e. it increases in the order *Gobio*, *Rutilus*, *Cyprinus*.

4. The mucus-secreting cells attain their maximum development in the

pharynx in all three fishes, as is usual amongst teleosts (cf. Al-Hussaini, 1947*a*, p. 278) and are thus effectively placed to lubricate the food, irrespective of its nature, at the very commencement of its journey through the gut.

5. Gill-rakers carrying taste-buds and mucus-secreting glands are present in all three fish, but they are exceptionally short in *Gobio* for a bottom-feeding fish. This may be compensated for to some extent by a pair of food-selecting palatal cushions.

6. All three fishes are stomachless, the short oesophagus joining the pharynx directly to the pyloric sphincter. The loss of the food-holding capacity of the stomach is compensated for by the swelling of the first limb of the intestine.

7. The intestinal tube is longest and its looping most complex in *Cyprinus*, and shortest and with the simplest looping in *Gobio*. Four parts are recognized in the intestinal tube, not by external features, but by their mucosal foldings and certain histological characters.

8. An estimation of the mucosal area shows that the absorptive area of the intestinal epithelium is practically equal in the three species when related to the weight of the fish. This ratio has here been called the 'mucosal coefficient'.

9. The intestinal epithelium comprises two principal histological cell types, viz. the absorptive cell and the goblet cell.

10. The internal surface across which food substances pass from the cells to the tissue fluids is greater in *Cyprinus* and *Rutilus* than in *Gobio*, owing to the greater length of the absorptive cells.

11. In order to be valid, estimations of the relative efficiency of the fish intestine should take into account the relative length of the gut (R.L.G.), the mucosal coefficient (Q.M.), and the length of the absorptive cells.

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EXPLANATION OF PLATES

Plate I

Fig. 1. *R. rutilus*, showing mucosal folds of roof of mouth, pharynx, oesophagus, and intestinal swelling.

Fig. 2. Transverse section of the buccal cavity of *Cyprinus carpio*. ×22. Note the abundance of taste-buds (T.B.) all over the cavity except on the dorsal surface of the maxillary valve (MX.V.).

Fig. 3. Tangential section of the lower lip of *Cyprinus carpio*, showing scores of taste-buds in cross-section. ×85.

Fig. 4. Transverse section of the epithelium lining the lateral part of the roof of the anterior pharynx of *Cyprinus carpio*. ×190.

Plate II

Fig. 5. Transverse section of the anterior pharyngeal region of *Cyprinus carpio*. ×26.

Fig. 6. Transverse section of the anterior pharynx of *Cyprinus carpio* passing through a gill arch. ×45.

Fig. 7. Pharyngeal 'jaws' of the three Cyprinids. ×2 approx. (*a*) the left 'jaw' of *R. rutilus* showing six teeth, medioventral aspect; (*b*) both 'jaws' of *R. rutilus* from the dorsal aspect; note asymmetry of teeth; (*c*) the right 'jaw' from the same specimen as (*a*), medioventral aspect; (*d*) the left 'jaw' of *Cyprinus carpio*, lateral aspect; (*e*) the right 'jaw' from the same specimen as the previous one, lateral aspect; (*f*) both 'jaws' of *G. gobio*, ventral aspect. In (*a*), (*b*), (*c*), and (*f*) the anterior end is pointing downwards.

Plate III

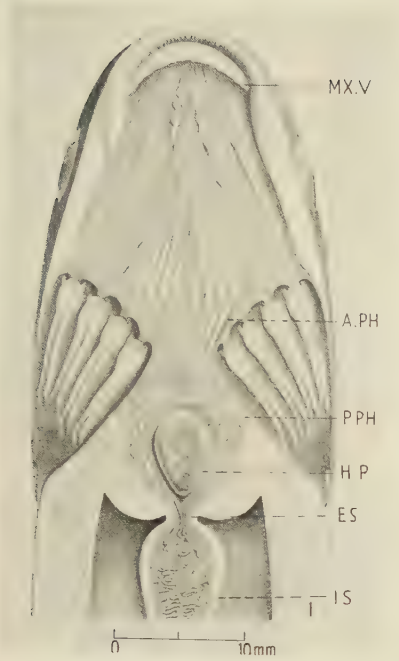
Fig. 8. Transverse section of the horny pad of *R. rutilus*. ×70. (*a*) drawing to show the cellular types composing the three zones of the pad.

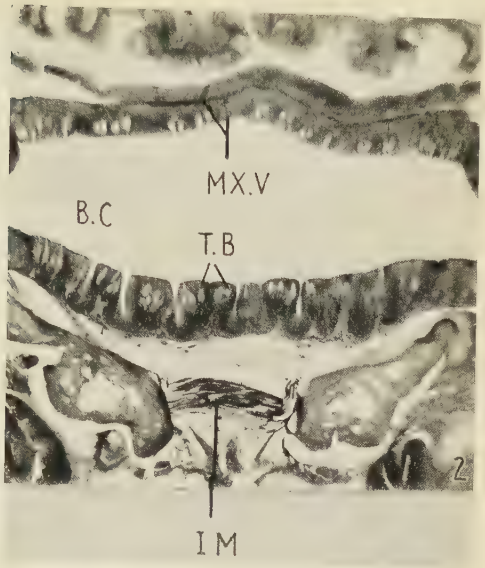
Fig. 9. Transverse section through oesophagus (ES.) & intestinal swelling (I.S.) of *R. rutilus*. ×20.

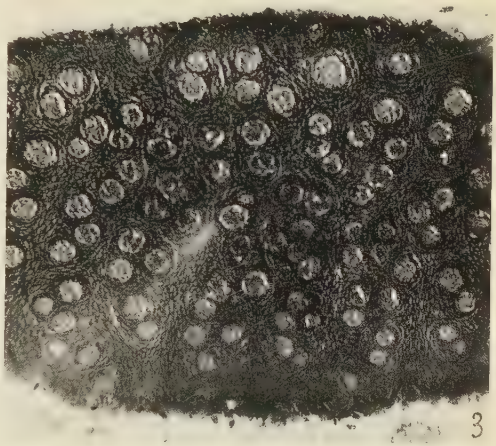
Figs. 2–6, 8, and 9 untouched photomicrographs; figs. 2–6 and 9, fixed Boling's fluid stained H & E; fig. 8, fixed Boling's fluid, stained Mallory's triple.

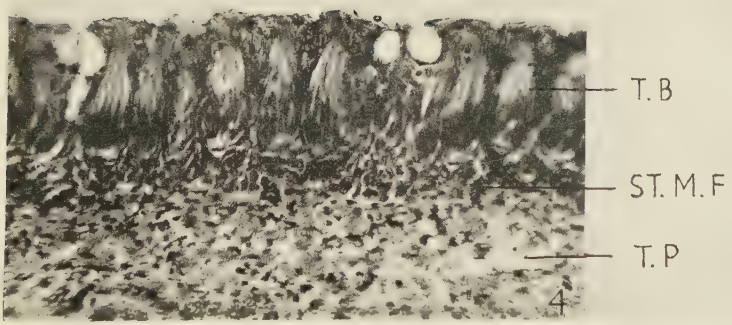
LIST OF ABBREVIATIONS USED IN THE ILLUSTRATIONS

A.B.	air-bladder.
A.PH.	anterior pharynx.
AN.	angular.
AR.	articular.
B.C.	buccal cavity.
B.M.	body musculature.
B.Z.	basal zone.
C.A.	coeliaco-mesenteric artery.
D.A.	dorsal aorta.
D.O.	dilator operculi.
DT.	dentary.
ES.	oesophagus.
GH.	geniohyoideus.
G.R.	gill raker.
H.P.	horny pad.
I.S.	intestinal swelling.
IM.	intermandibularis.
IN.	intestine.
I.O.	interopercular.
L.	ligament between maxilla, premaxilla, and dentary.
L.A.P.	levator arcus palatini.
LV.	liver (hepatopancreas).
M.C.	muscularis circularis (forming sphincter).
M.L.	muscularis longitudinalis.
M.P.	masticatory process of basi-occipital.
M.S.C.	mucus-secreting cells.
M.Z.	middle zone.
MD.AD.	mandibular portion of adductor mandibulae.
MX.	maxilla.
MX.AD.	maxillary portion of adductor mandibulae.
MX.V.	maxillary valve.
OP.	opercular.
P.D.	pneumatic duct.
P.PH.	posterior pharynx.
PH.C.	pharyngeal cavity.
PL.	palatine.
PMX.	premaxilla.
POP.	preopercular.
R.A.B.D.	retractor arcus branchialis dorsalis.
R.L.	rostral ligament.
S.Z.	superficial zone.
SH.	sternohyoideus.
SOP.	subopercular.
ST.M.F.	striated muscle-fibre.
T.B.	taste-bud.
T.P.	tunica propria.
TH.G.	thymus gland.
TZ.	trapezius.

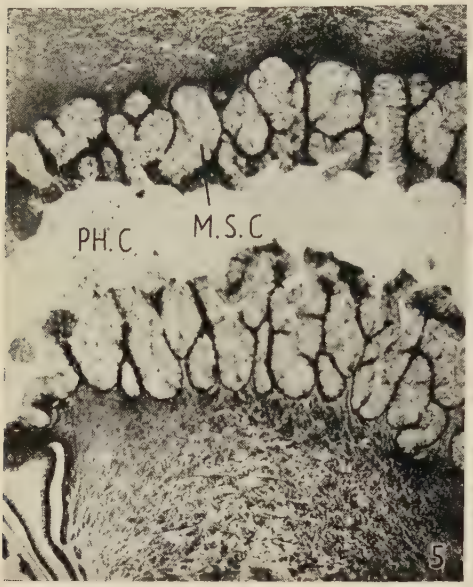


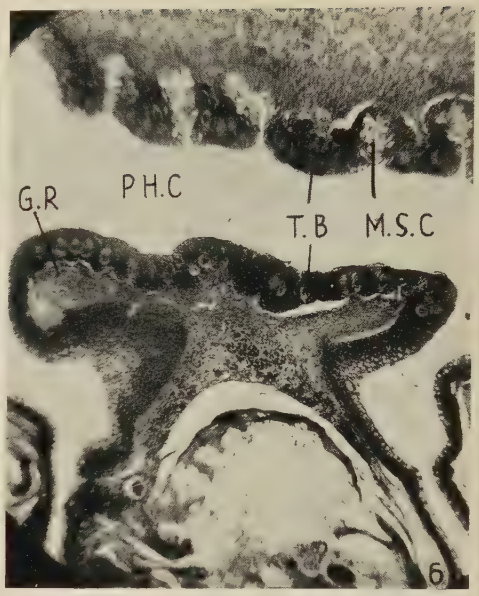


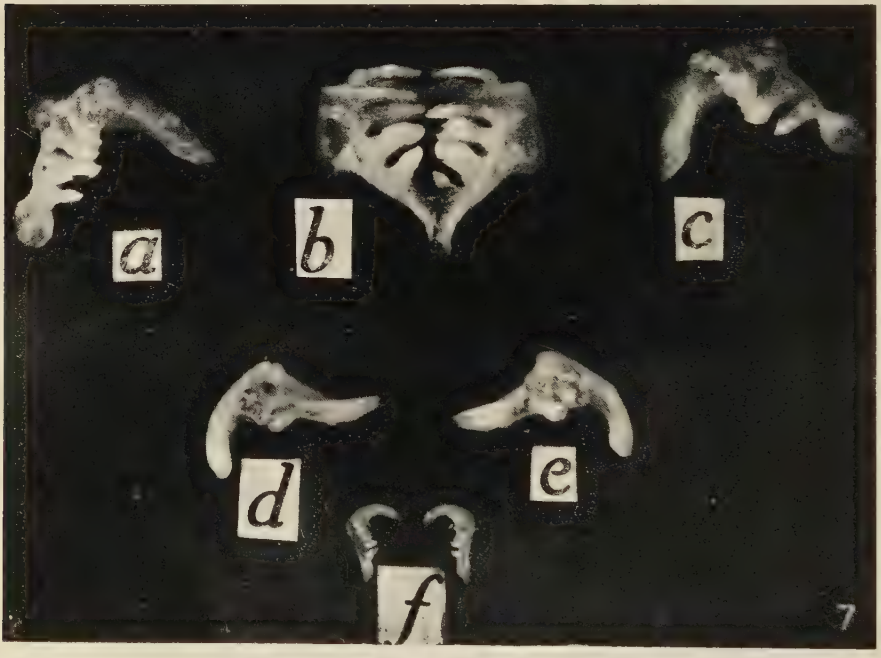




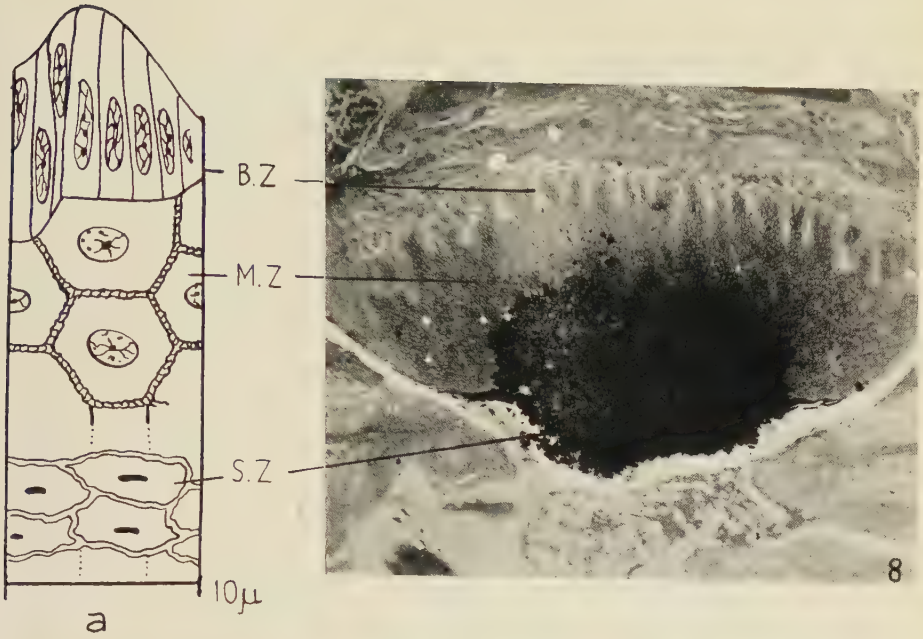
AL-HUSSAINI—PLATE I

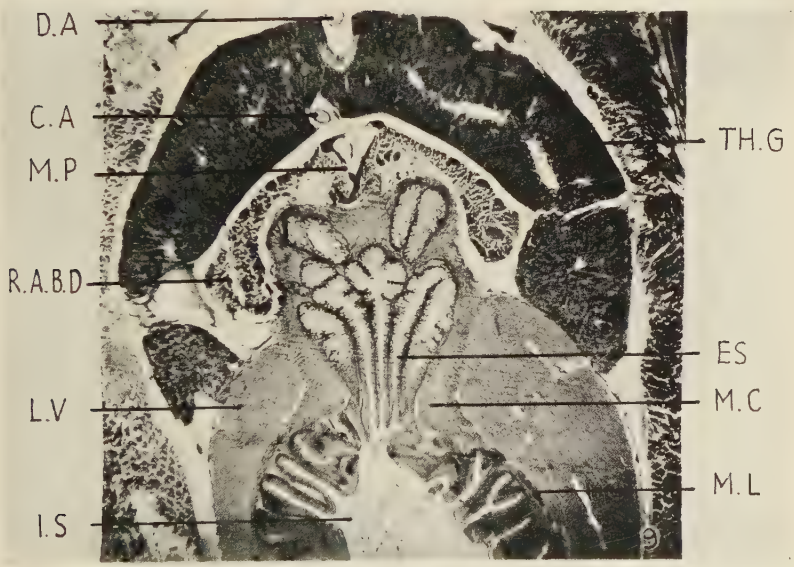






AL-HUSSAINI—PLATE II





AL-HUSSAINI—PLATE III

Observations on Hypotrichous Ciliates: The Genera *Stichotricha* and *Chaetospira*

BY

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CONTENTS

INTRODUCTION

WHILE examining the empty cells of certain pondweeds, I became interested in two hypotrichous ciliates that occurred there with some regularity and often in considerable numbers. The one that drew my attention first was a loricate organism that I identified as a species of *Chaetospira* Lachmann (1856). The other was a non-loricate form which was much less modified for a restricted habitat, and migrated from cell to cell even when adult. This second organism answered to the description of *Stichotricha* Perty (1852), although its morphology did not precisely accord with that of any of the species recognized by Kahl (1932).

Whereas the free-swimming scavenging Hypotricha, such as *Euplotes* and *Stylonychia*, have been exhaustively studied, little has been recorded of the behaviour and reproduction of the cell-inhabiting species, although the restriction on their movements makes them comparatively easy to observe for long periods at a time. The following notes, though fragmentary, may serve to fill some gaps in our knowledge; and they may also help to clear up some taxonomic confusion. After a brief historical survey, I propose to deal first with *Stichotricha*, since it is the more comparable with 'normal' hypotrichs: more precise information as to its structure, life-history, and behaviour undoubtedly helps in the interpretation of the highly modified *Chaetospira*.

HISTORY

The confusion in the nomenclature of these genera is almost impossible to resolve. *Stichotricha* was first described in 1852 by Perty, 'lancelet-shaped with the extensible anterior end narrow, flat, drawn out and carrying the peristome. On one side of this is a row of large obliquely-standing cilia.'

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Although his description was so incomplete that the ciliate 'could have been a *Loxodes* or *Amphileptus*' (Lachmann, 1856), Perty's name *Stichotricha secunda* for the type species has been retained. He placed it in the Oxytrichina. Four years later Lachmann (1856) described two species of a new ciliate genus *Chaetospira*, *C. mülleri* and *C. mucicola*, both of them freshwater and loricate. Of this genus he wrote, 'when extended the proboscis (Rüssel) forms more than one turn of a spiral and the first membranelle is longer and stronger than the rest'. He placed *Chaetospira* near *Stentor* but realized that it was related to *Stichotricha*. In 1862 Strethill Wright described, but did not figure, another species of *Chaetospira*, which he named *C. maritima* on account of its marine habitat. A third freshwater species, *C. remex*, was described by Hudson (1875); but this, together with Bolton's *C. cylindrica*, recorded in 1878, was relegated by Savile Kent (1880) to the genus *Stichotricha*. Gruber (1879) had already said that *Stichotricha* and *Chaetospira* were identical, and his *S. urnula*, described in 1883, certainly is very closely related indeed to *Chaetospira* and possibly forms a bridging species between the two genera. Entz (1884) again identified *Chaetospira* and *Stichotricha* and said that his newly described *S. inquilinus* was none other than *C. mülleri*. Möbius (1888) recorded *C. maritima* from the Kiel Canal and figured it. Meanwhile eight other species of *Stichotricha* had been described. Some of these are possibly species of the genus *Chaetospira*.

Another complication was introduced by Sterki (1897), who, while giving the most complete description as yet of *Chaetospira mülleri*, seems not to have known of this genus. He called his ciliate *Spirotricha paradoxa*, and he appreciated the differences between it and *Stichotricha*. Kahl (1932) returned Sterki's *Spirotricha*, together with a variety *S. paradoxa univacuolata* Illowaisky (1913), to the genus *Chaetospira*, species *mülleri*. In the same work Kahl recorded *C. entzi* as another bridging species between *Chaetospira* and *Stichotricha*, the thirteen described species of which he reduced to eight.

Material and Methods

Chaetospira was first found by me on algal encrustations at water level on the sides of a shallow glass jar of pond water in which were growing *Lemna*, *Cladophora*, and *Riccia*. The sources of the material were not recorded. Later collections were made from ponds in or near London. These never yielded cysts as did the first batch, and the *Chaetospira* were always found in the dead cells of *Lemna trisulca* or *Riccia fluitans*. Once they occurred in the root of *Lemna minor* and once in the dead cells of some unrecognizable plant debris.

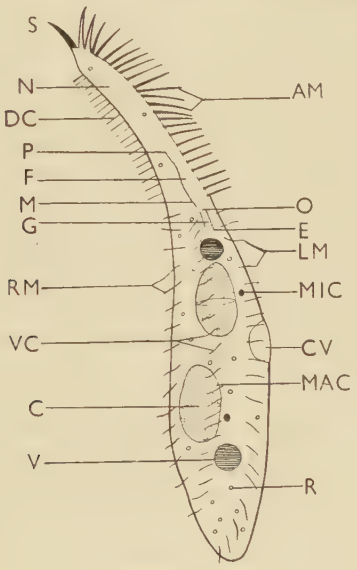
Stichotricha was found inhabiting the empty cells of leaves of *Lemna trisulca* and roots of *L. minor*. These two species of *Lemna*, collected over a period of 18 months, were kept in tongue jars in the laboratory.

Most of the observations were made on living material and consisted in watching behaviour, division, and, in *Chaetospira*, lorica formation. Certain individuals of this genus could be watched for several weeks; but *Stichotricha*

rarely stayed in the same cell for more than a day. However, this was long enough for all the stages of asexual reproduction to be followed.

In feeding experiments the animals were placed in pond water enriched with bacteria and small undetermined flagellates, or in a carmine suspension.

For cytological purposes *Lemna* leaves or roots having a fair ciliate population were fixed in alcoholic Bouin or Champy, Zenker or Schaudinn and stained with Heidenhain's iron haematoxylin, haematein (after Dobell),



Text-fig. 1

Delafield's haematoxylin, or haemalum by the Vicelle method. This method I had direct from Professor Hovasse, who recommended it for demonstrating micronuclei: after fixation wash overnight in tap-water, rinse in distilled water, and stain in a very weak aqueous solution of haemalum for 24 hours. Feulgen following Champy was frequently used.

Stichotricha Intermedia n. sp.

Trophic Phase (Text-fig. 1)

This species, like others of the genus, has a contractile, flexible, spindle-shaped body, drawn out anteriorly into a proboscis (N), usually bent but never spiral, bearing along its left border a row of powerful adoral membranelles (AM). The whole animal is slightly spirally twisted especially when extended in feeding (Text-fig. 2, fig. 1). The terminal membranelle is larger and set apart (S). Ventrally there run three longitudinal rows of cilia curving posteriorly to the right (RM, LM, VC). At the base of the proboscis lies the mouth (O) and to the right of it a hyaline ectoplasmic membrane (M). There are two

macronuclei (MAC), and the contractile vacuole (CV) is dorsal and on the left, roughly half-way along the length of the body.

In the adult of this species, length varies from 40 to 170 μ: when extended about two-fifths is occupied by the proboscis. It is characterized by two rows of dorsal hair-like cilia (DC), all the other species, according to Kahl (1932), having three rows. There are in this species, in *S. secunda* (for a full account of ciliation in *S. secunda* see Stein, 1859), and probably in all others, a row of fine, stiff, seta-like cilia supporting the ectoplasmic membrane on the right of the peristome. These form a parabolic curve (P), and, following Sterki's nomenclature for similar structures in *Spirotricha* (=*Chaetospira*, 1897), they may be called the paroral cilia. The membrane, together with its cilia, runs back to the mouth from the point where the proboscis is bent. Endoral cilia (E) line the gullet.

The pellicle is very thin. There is no differentiation into ectoplasm and endoplasm, but the cytoplasm of the proboscis is hyaline and contains only a few greenish, refringent granules (R). These are more numerous in the main mass of the body, where also lie the nuclei, contractile and food vacuoles (V). The contractile vacuole, which at diastole projects on the left dorsal surface, contracts every 15–20 seconds—at more frequent intervals in young than in older individuals. The anus is mid-dorsal and the creature emerges from the sheltering cell just enough to allow the faecal material to be deposited outside (Text-fig. 2, fig. 1 A).

Perty (1852) did not mention the nucleus. Kahl (1932), in his monograph on Ciliates, says of the nucleus of *Stichotricha* only that it is in two pieces. Obviously this refers to the macronucleus. The two lie in tandem, each an ovoid structure with its long axis parallel with that of the ciliate. In the living organism the macronucleus appears as a refringent greenish body. About half-way along its length is a transverse cleft. In the preserved ciliate the macronucleus is made up of a chromatin network surrounding a number of vacuole-like spheres (cf. Willis, 1942, and Wenrich, 1929*b*). Embedded in the side of, or somewhere near, each macronucleus is a micronucleus. This stains densely and uniformly in interphase, but is not visible in the living animal.

Behaviour

The species of *Stichotricha* under consideration is intermediate in its habits between the freely swimming *S. secunda* and the loricate *S. socialis*. Unlike the former it may remain in one particular cell for several days, but never inhabits the same cell permanently as does *S. socialis*.

When feeding, the animal is extended (Text-fig. 2, fig. 1), the proboscis projecting beyond the aperture of the sheltering cell and bending to one side, the posterior end of the body being braced to one or more sides of the cell wall by the posterior cilia.

When disturbed the animal withdraws rapidly to the inmost corner of the cell, moulding its shape to fit against the wall. At the same time the proboscis

is contracted and thickened but never retracted into the body (cf. *Chaetospira*). Withdrawal is brought about by the adoral membranelles. The ciliate usually emerges again quickly and continues feeding. It may, however, remain withdrawn for a longer time, especially if environmental conditions are unfavourable, e.g. increased temperature or gradual desiccation. These conditions, amongst others not yet determined, also cause the migration of the animal to a new site. The behaviour of the ciliate during migration is the same as that of the swarmer after asexual reproduction, and is dealt with in the following section.

Stichotricha usually feeds on bacteria and other small organisms. Occasionally it will swallow larger organisms such as coloured flagellates. *Chlamydomonas* spp. measuring 7 μ in diameter were fed to these *Stichotricha*, and even though the flagellates were as broad as the proboscis (i.e. three times the diameter of the gullet), they were ingested (Text-fig. 2, fig. 3). As there are no records of feeding methods in *Stichotricha*, a brief account based on my observations may be of interest.

A feeding current is promoted by the adoral membranelles and is sufficiently powerful to bring in particles from some considerable distance.

Small particles, about 1 μ in diameter, are ingested indiscriminately if they get carried to the food groove. Larger particles, 7–10 μ in diameter (width is the important dimension), are undoubtedly selected. Particles of carmine and faecal debris of this size are always rejected. Flagellates are always accepted.

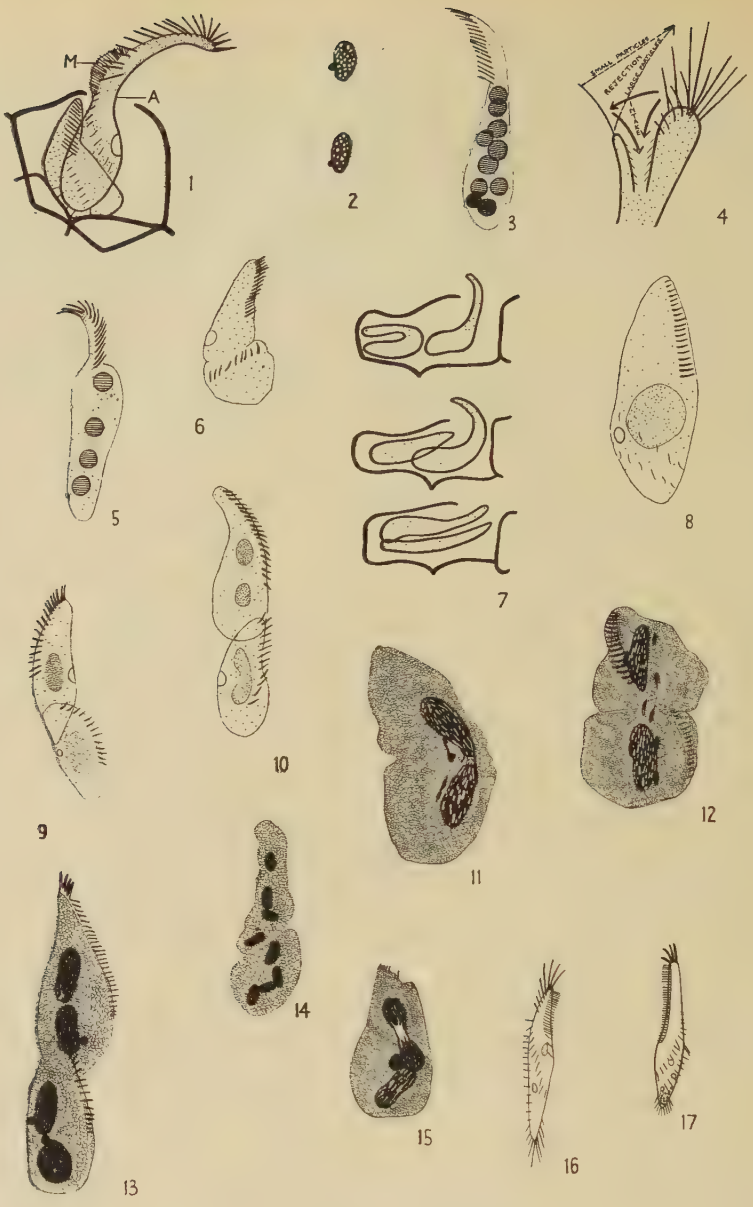
Playing an important role in selection is the membrane and its paroral cilia. These are so placed in relation to the row of adoral membranelles that a V-shaped food groove lies between them, its floor formed by a strip of proboscis cytoplasm running the length of the membrane and back to the mouth. Normally the membrane is held stiffly, and curves outward gently from the base of the food groove directing the current. In doing this it plays a passive role (Text-fig. 2, fig. 4).

When a large edible particle is brought into the food groove, the membrane, moved by the parorals, closes over it and appears to push it down into the gullet. With very large edible particles, such as the *Chlamydomonas* already mentioned, strenuous gulping movements of the cytoplasm take place as in *Stokesia* (Wenrich, 1929*a*). While the monad is trapped by the membrane and is revolving in the food groove at the base of the peristome, the cytoplasm within the body surges forward and surrounds it. The combined efforts of the displaced cytoplasm and the membrane finally bring about ingestion (Text-fig. 2, fig. 5). After this the cytoplasm that had piled up at the base of the proboscis returns to its normal position.

Rejection of large particles is accomplished by spasmodic jerks of the membrane.

Asexual Reproduction

Preparatory stages. Animals about to divide by binary fission have not necessarily reached a maximum size. Whereas the ciliate in Text-fig. 2, fig. 1,



TEXT-FIG. 2. *Stichotricha intermedia* n.sp.

(Except where otherwise stated, drawings, made with aid of camera lucida, are of living specimens × 360.)

1. Left side view of adult feeding. Contracted position also shown. M, ectoplasmic membrane. A, position of anus. 2. Nuclear apparatus of adult. × 800. Feulgen. 3. Specimen that has fed well on algae. 4. Diagram of proboscis seen end on at the bend, showing feeding currents. 5. Displacement of cytoplasm in swallowing a large food particle. 6. Onset of cytoplasmic fission. 7. The products of fission after separation but before migration. Freehand. 8. The two macronuclei have fused prior to fission. 9. After fission but before separation, each daughter ciliate with a single macronucleus. 10. The single macronucleus of each daughter dividing. 11. The fusion macronucleus and two micronuclei dividing during binary fission. Haemalum. × 800. 12. The macronucleus and two micronuclei of each daughter dividing. Haemalum. × 800. 13. The two macronuclei of each daughter ciliate dividing. Haemalum. × 800. 14. Each daughter ciliate with four macronuclei. Haemalum. 15. The two macronuclei of a daughter ciliate dividing after separation. Haemalum. × 800. 16. *S. simplex*, after Kahl. 17. *S. gracilis*, after Möbius.

has grown to 96 μ before dividing, that in fig. 6 has divided when only 52 μ in length. Other workers have found that the rate of multiplication in ciliates may be independent of size (Harding, 1937, and Chatton and Beauchamp, 1923). Adolph (1931) came to the conclusion that 'when the processes which have to do with age come to a certain point, fission occurs regardless of how much body substance is present'. In the case of the organism described here it may be that the size of the containing cell in some way determines division. The onset of fission is marked by the withdrawal of the ciliate and consequent cessation of feeding, whereas other Hypotricha are active during division. Beating of the posterior adoral membranelles continues.

Cytoplasmic fission. Fission begins on the oral side just behind the peristome and extends by an oblique line across to the dorsal side behind the contractile vacuole (Text-fig. 2, fig. 6). The posterior half acquires new organs, whereas the anterior half retains those of the parent, as in *Euplotes harpa* (Wallengren, 1901). The new membranelles appear before cleavage is complete, but in what order I am unable to say. In addition a contractile vacuole is working in the posterior half by this time. While the line of cleavage deepens, the dividing ciliate rotates from time to time and may slowly expand and contract. When fission is complete, the two resulting ciliates remain one behind the other for some time, their membranelles beating actively. About 10 minutes later the posterior individual or swarmer moves up towards the opening of the cell and lies alongside (Text-fig. 2, fig. 7) or in front of the anterior individual, and it is difficult to distinguish one from the other. The whole process occupies about 2 hours.

Fate of the swarmer. The two individuals produced as a result of binary fission may remain together within the same cell, providing there is sufficient room for both to protrude and feed. If this happens, then the anterior one extends its neck and begins to feed shortly after fission, whereas the swarmer spends a preliminary period within the enclosing cell, extending and retracting, rotating and 'exploring'. After this it partially emerges and commences feeding. This remaining together of the products of fission sometimes results in groups of four individuals; but these are independent and not alined as in *S. socialis* (Gruber, 1879).

Migration and settlement. It is more usual for the swarmer to migrate after division. Before doing so it makes excursions to the aperture of the cell, pushing its sister ciliate back. These exchange movements continue for some time, the swarmer seeming to become bolder and emerging tentatively through the cell opening. Finally, it squeezes the broader posterior end of its body through and escapes. Its behaviour now depends upon local conditions.

If there is plenty of *Lemna* and it comes into contact with this, it will crawl over the plant in a persistent and exploratory manner, searching for a possible settling place. It does this by means of the membranelles and the few body cilia. The crawling is a jerky progression in straight lines, alternating with sudden backing movements brought about by a reversed beat of the membranelles. In backing the front part of the body is bent on the rest and

then straightened suddenly so that the ciliate points in a new direction in which it now proceeds. The front end is continually poking into crevices and empty cells. Sometimes these will be entered. Feeling all round the inside of the cell, using the larger anterior membranelles, projecting the anterior end through the aperture of the cell as if testing the nature of the surrounding water, and then backing and twisting round inside, the swarmer seems to test thoroughly the new abode. It may remain here or leave and begin the process of trial again. No feeding is done during this migratory period. As with Hypotricha in general and the swarmers of the Peritricha, the ciliate at this stage is markedly thigmotactic.

If, however, there is not much *Lemna*, and the swarmer swims straight out into open water after binary fission, then its behaviour is different. The swimming is continued. This is a rapid forward spiralling movement brought about by the membranelles and by the fact that the body is lop-sided owing to the disposition of the curved proboscis. As a result of the spiralling a much larger volume of water is explored. Although the movement is usually forward, reversals are not infrequent, and the bending of the front end, with subsequent change in direction, results sooner or later in some solid surface being contacted. Then the crawling and exploring movements begin.

The duration of this free-swimming phase varies from one to many hours (cf. *Chaetospira*).

Cytology. The behaviour of the macronuclei can be followed in the living ciliate. Fusion of the two macronuclei takes place before the cytoplasm cleaves (Text-fig. 2, fig. 8). Shortly after fission has started, the fusion nucleus divides into two, one of which passes forwards into what will be the anterior individual, while the other takes up its position in the swarmer (Text-fig. 2, fig. 9). Before fission is completed each macronucleus divides again. The products of this division, however, do not become the definitive nuclei of the new individuals, for they are seen to undergo the initial stages of a further division before the daughter ciliates separate. This division may be completed before or after separation (Text-fig. 2, figs. 14 and 15). Presumably the four macronuclei now in each product of division fuse two by two to form the two adult macronuclei, the line of fusion being represented by the cleft characteristic of hypotrichous nuclei.

The behaviour of the micronuclei can be followed only in stained material. While the fusion macronucleus is dividing, the two micronuclei divide to form four and then eight. The fate of these is obscure for only two persist in each daughter ciliate. Perhaps as in *Kahlia simplex* (Horvath, 1936) some of them disintegrate and do not participate in the final stages of fission (Text-fig. 2, figs. 11, 12, and 13).

While dividing, the chromatin of the macronucleus is in the form of beaded threads (Text-fig. 2, fig. 14), short and thick in the early and late stages, long and attenuated in the middle stages. These threads are so numerous and crowded together that it is impossible to count them. The same is the case with the micronuclei; and here extremely small size adds to the difficulty.

TABLE I. *Showing Chief Distinctive Structural Features of Nine Stichotrich Species*

(.. indicates no record)

SPECIES	*secunda*	*marina*	*aculeata*	*socialis*	*saginata*	*gracilis*	*opisthotonoides*	*simplex*	*intermedia n.sp.*
FOUNDER	Perty	Stein	Wrzesniowski	Gruber	Möbius	Möbius	Smith	Kahl	Froud
DATE	1852	1867	1870	1880	1888	1888	1897	1930	1944
HABIT	Solitary	Solitary	Solitary	Social	Solitary	Solitary	Solitary	Solitary	Solitary
HABITAT	*Chara* & *Myriophyllum*	*Ulva*	*Sphagnum*	Flooded meadows	*Ulva*	..	Old infusions	..	*Lemna*
LORICA	Absent	Absent	May or may not be present	Gelatinous	Absent	Absent	Absent	Absent	Absent
LENGTH IN μ	130–200	180–260	100	200	200	100	55	85–120	40–170
PROBOSCIS	Bent	$\frac{1}{8}$ body length	Straight $\frac{1}{4}$ body length	Bent $\frac{1}{2}$ body length	Thick	..	Bent dorsally	$\frac{1}{3}$ of body length	Bent $\frac{2}{5}$ body length
FRONTAL CIRRI	..	..	..	3–4	..	5	..	5	None
ROWS OF BODY CILIA	4	4 ridged	4	4	4	2	..	2	3
LENGTH OF DORSAL CILIA IN μ	20	7	..	..	..	..	..	7	5

Systematic Position

Of the eight species of *Stichotricha* described by Kahl (1932), *S. simplex* (Text-fig. 2, fig. 16) agrees most closely with the one described here (see Table I). Both have three rows of cilia on the narrow, spindle-shaped body and the proboscis occupies a third of the body length. Both are non-loricate and, though widespread, are not profuse. The size range of *S. simplex*, 85–120 μ in length, is comparable with that for my species, but it has five frontal cirri whereas mine has what might be called a frontal cirrus, but this is really the first membranelle, which sticks out horn-like as does that in *S. secunda*. The dorsal cilia of *S. simplex* measure 7 μ as compared with 5 μ in the new species. These are only small differences, and it might be considered that my species is identical with *S. simplex*; but it does not seem wise to give it the same name until more is known about them both.

Again it is possible that the species described here is identical with *S. gracilis* Möbius, sketched by him in 1888. This species also shows reduction of body ciliation (Text-fig. 2, fig. 17) although Kahl (1932) attributes this to oversight in observation. No account of *S. gracilis* was given. If it does have only two rows of body cilia (see Table I), then it has gone farther in the process of cilium-reduction than my species, which is intermediate between it and the more typical species. Here again, until more is known about *S. gracilis*, it seems best not to identify mine with it.

S. opisthotonoides (see Table I) is also so incompletely described that it is impossible to say whether I have been dealing with it rather than with a new species. Like mine it is freshwater, solitary, naked, and has a bent proboscis. Its small size (55 μ) might well be accounted for by the fact that the specimens measured by Smith (1897) were all young ciliates, in which case they would agree with the species described here.

On these grounds a new species is proposed for this *Stichotricha*, and because several of its characters are intermediate between those of other species of the genus it is called *S. intermedia*.

The following is the diagnosis of *Stichotricha intermedia*:

Systematic position. *Stichotricha intermedia* n.sp. (Spirotricha, Hypotricha, Oxytrichidae).

Description. Solitary; non-loricate; length of adult 40–170 μ, two-fifths of which is occupied by a bent proboscis; three rows of body cilia; dorsal cilia 5 μ in length, two rows.

Habitat. *Lemna*.

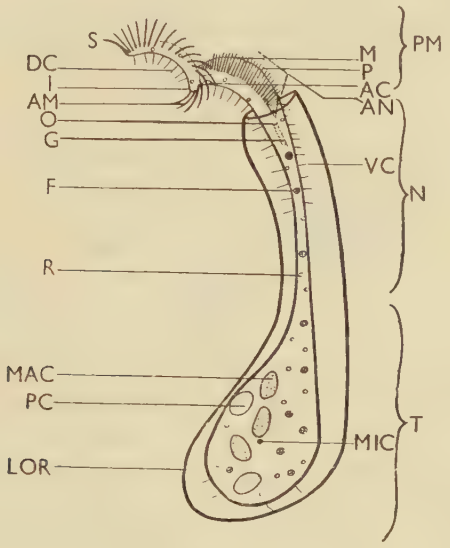
Locality. London, England.

Chaetospira mülleri Lachmann

Trophic Phase (Text-fig. 3).

Chaetospira is a flask-shaped organism varying in length from 60 to 200 μ. When feeding, the neck of the flask is drawn out into a spiral (PM), which may form as much as a third of the length of the body, and runs in an

anti-clockwise direction. On this proboscis are borne the powerful adoral membranelles (AM). Of these the first few are stouter and more widely separated than the rest. The first (S) in particular is very conspicuous, differing from the others in size, appearance, and behaviour. In addition to the cilia of the membranelles there are accessory peristomial cilia (VC), stiff, sensory dorsal cilia (DC), and a small undetermined number of body cilia. Opposite the adoral membranelles on the left-hand margin of the peristome and running from the first twist of the spiral back to the mouth (O) is a thin



TEXT-FIG. 3

ectoplasmic membrane (M) supported by a series of stiff paroral cilia (P). The mouth lies at the base of the proboscis and leads into a gullet (G) which penetrates the neck. This and the proboscis are composed of hyaline cytoplasm containing a few of the refringent granules which in a well-nourished specimen fill the base of the flask-shaped body (T). This contains the contractile vacuole (PC), food vacuoles (F), and the nuclei (MAC, MIC). There may be another contractile vacuole (AC) on the proboscis, left and dorsal to the adoral membranelles, on the first turn of the spiral. As in *Stichotricha* the pellicle is very thin and the body is plastic, without any real distinction between ectoplasm and endoplasm. The anus is far forward on the proboscis, dorsal, to the left and a little way behind the first turn of the spiral (Text-fig. 4, fig. 18).

Hitherto the nucleus of *Chaetospira* has not been adequately described. In Sterki's words, 'Two almost globular endoplasts were seen not very distinctly.' Kahl, in 1932, said that the nucleus of *C. mülleri* is in two separate pieces. Both these remarks refer to the macronucleus. Now my species of *Chaetospira* has its macronucleus in rounded pieces, usually four, as in *Gastrostyla*

(Weyer, 1930). But as Lachmann, the discoverer of *C. mülleri* (with which species I think I am dealing), did not mention the nucleus, there is no justification for setting up a new species until the cytology of the genus has been studied.

In the living animal nuclei cannot be seen. Feulgen-stained specimens (Text-fig. 4, fig. 20) show the macronucleus to be made up of a chromatin network surrounding a number of vacuole-like spheres as in *Stichotricha*. The number of macronuclei in trophic individuals varies from two to eight

TABLE II. *Showing that Macronuclear Number is independent of Body Length in* C. mülleri (*fixed material*)

No. of macronuclei	8	6	5	4	3	2	1
Body length in μ of individuals	31	32	30	32	40	35	35
	54	42	32	41	69	72	65
		57	42	54			
			54	72			
			69	91			
			128	111			
				132			

(cf. *Urostrongylum contortum* Kahl (1932)). There is no correlation between the number of macronuclei and the size of the individual (see Table II). This may be accounted for by one or more of the following assumptions:

1. Endomixis might be occurring in these apparently ordinary individuals. Against this is absence of disintegrating macronuclei.
2. Mitosis might be completed before cytoplasmic fission starts. Against this is the rareness of division figures; but supporting it are the individuals of all sizes with five to eight nuclei.
3. In many ciliates, e.g. *Stentor*, the adult macronucleus is the product of several 'Placenten' (Bělař, 1926). This might account for the large number of macronuclei in some of the small ciliates, and for what looks like the fusion of macronuclei in pairs in a few cases.

The micronucleus is usually single, but at times two or more have been seen. It lies near or in a notch in the side of one of the macronuclei (Text-fig. 4, fig. 21). In interphase it is densely and uniformly stained. Consequently when it lies above or below a macronucleus it cannot be seen. This may account for the variation in number recorded above. On the other hand this might be due, as in the case of the macronucleus, to precocious mitosis.

Feeding

Chaetospira feeds on the same kinds of food and in the same way as *Stichotricha*. I have seen it ingest *Bodo*, diatoms, and considerable lengths of algal filaments. Sterki (1897) observed this and noted also that the posterior adoral membranelles are responsible for rejection. This I have not seen. The

TEXT-FIG. 4. *Chaetospira mülleri*

(*Except where otherwise stated, drawings, made with aid of camera lucida, are of living specimens* ×360.)

18. Diagrammatic sketch of proboscis, ventral view, showing three successive positions of a faecal pellet about to pass out through the anus A. E, ectoplasmic membrane. 19. Retraction of proboscis in a contracted animal. 20. Nuclear apparatus of adult. ×580. Feulgen. 21. Fusion macronucleus prior to fission. ×580. Haemalum. 22. Dormant phase prior to fission. 23. Cytoplasmic fission. 24, 25, and 26. Different appearances of the products of fission after separation but before migration. Freehand. 27. Swarmer exploring a new site. 28. The nuclear picture immediately after fission. Haemalum. ×800. 29. Formation of lorica: vacuolation. 30. Same: withdrawal. 31. Neck of new lorica before it is open. Freehand. 32. Adding new length of neck to lorica: vacuolation. 33. Same: withdrawal. 34. Cyst. 35. Same, stained Heidenhain's iron haematoxylin. ×580. 36. Group of feeding, resting, and encysted individuals. ×72.

membranelles can be seen moving only during extension or retraction of the proboscis and at certain times during asexual reproduction. At other times their rate of beating is so rapid that they appear motionless. I could see no differential beating of the posterior ones such as one would expect if they were the rejecting agents.

In a large, fully extended, feeding *Chaetospira*, food vacuoles are formed every 20 seconds. If cysts are ingested, the contents are utilized and the empty cases egested. The colour of a diatom disappears 10 minutes after ingestion, and *Bodo* is immobilized in less than 1 minute.

Asexual Reproduction

Preparatory stages. Individuals about to divide by binary fission are large and have their cytoplasm packed with greenish granules. The first sign of division is the withdrawal of the proboscis and rounding of the anterior end of the now pear-shaped body. Because the adoral zone is contracted, no feeding is done and the creature is sluggish, but the posterior membranelles continue to move though slowly and discontinuously. When not moving they are closely pressed to the body. This dormant period lasts for several hours (Text-fig. 4, fig. 22).

Cytoplasmic fission. Fission takes place as in *Stichotricha* (Text-fig. 4, fig. 23). All the dividing ciliates that I watched were in loricas and not easy to observe on account of the disposition of the sheltering cells, so that I was not able to determine how or when the ciliation of the posterior individual is laid down. It is this posterior half that acquires new organs, whereas the anterior half retains those of the parent. While the two new ciliates are still attached by a strand of cytoplasm dorsally, the membranelles of both begin to beat, but no attempts are made at this stage to emerge and feed. After some time separation is effected and the two inhabitants of the lorica now roll round one another, frequently changing places (Text-fig. 4, figs. 24 and 25).

Escape of the swarmer. The behaviour of the two individuals is different. The membranelles of the posterior individual or swarmer are less well developed than those of the anterior. They appear ragged and beat haphazardly so that the swarmer makes jerky movements. It will come up to the base of the neck of the lorica, stick out its now pointed anterior end, and then withdraw rapidly to the back end of the lorica. When it does this the anterior individual is first displaced from the neck end and retires, then moves forward again when the potential swarmer withdraws. The movements of the anterior individual with its clear-cut membranelles are more regular; and after a time it extends its proboscis at intervals through the lorica neck and feeds (Text-fig. 4, fig. 26). Feeding is interrupted when the swarmer makes its forward exploratory movements. Sometimes both are forward together (Text-fig. 4, fig. 25).

Not for long do the two remain in one lorica. They are too cramped and it is impossible for them both to feed. The swarmer comes more and more frequently to the lorica neck, pushing the other back: it makes more and more

frequent essays at extending its proboscis through the lorica mouth, and finally squeezes out through the neck and swims away. The anterior individual is now left in possession of the lorica. It immediately proceeds to feed and carries on as a typical trophic *Chaetospira*.

Migration and settlement. The swarmer acts as a distributive phase. It is still unlike the adult in that the proboscis is relatively undeveloped, with few membranelles and not spirally twisted but bent over to one side as in *Stichotricha*. The size of the swarmer varies with that of the parent, but an average length of 46 μ was obtained from fifteen specimens. On leaving the parent lorica the behaviour of the swarmer is very similar to that of *Stichotricha* (Text-fig. 4, fig. 27).

The duration of this free-swimming phase varies. In one case, escape from the parent lorica was followed by immediate contact with a leaf of *Lemna*, discovery of an empty cell 5 minutes after, and its exploration and acceptance 10 minutes after this. In another case, the free-swimming ciliate was watched for half an hour before it finally settled.

I must remark here upon the striking resemblance between this distributive organism and the corresponding stage in *Stichotricha*. Morphologically they are identical except for small details in ciliation and in the nature of the nucleus. The *Chaetospira* swarmer is never free for such long periods as that of *Stichotricha*. As regards adaptation to a loricate existence, *Chaetospira* has gone much farther than the other ciliate, and the adult is considerably modified accordingly. But here is an example of a 'young' stage, retaining during its development the facies of a less-specialized genus of the family. Possibly this resemblance has been responsible for some of the confusion of the two genera, observers having mistaken swarmers of *Chaetospira* for free-swimming individuals of *Stichotricha*. In view of this some revision of stichotrich species seems necessary.

Cytology. Owing to the innumerable greenish granules present in the cytoplasm during division, it is impossible to follow nuclear behaviour in the living organism. Consequently my observations have had to be made on preserved material. This is unsatisfactory for the nuclear pictures are so variable that I am as yet at a loss to explain many of them. All I can say at present is that I believe the following to happen during binary fission:

The macronuclei fuse (Text-fig. 4, fig. 21). The fusion nucleus divides into two, four, eight, or more, usually. Cytoplasmic fission separates an anterior individual with four macronuclei from a posterior one with eight (Text-fig. 4, fig. 28). These eight fuse in pairs forming the four adult macronuclei. The fusions are not necessarily simultaneous. Moreover, they can take place before or after the daughter cells have separated. The micronuclear cycle has not been followed.

Lorica Formation.

The adult lorica (Text-fig. 4, fig. 36). This is flask-shaped, with its base embedded in an empty plant cell and its neck protruding from the opening.

It is thin and perfectly smooth. I can find no evidence for its being sticky, except perhaps when it is first laid down. The accumulation of debris around the neck of the flask, described by Möbius (1888) and Sterki (1897), is due to the method of feeding: for in *Stichotricha*, where there is no lorica but the same mode of feeding, the debris collects round the opening of the cell sheltering the animal. In both these ciliates rejected solid matter tends to be deposited where the quickly travelling water-current meets the static pond water. This is outside the ambit of the membranelles at the base of the proboscis and, in *Chaetospira*, on a level with the mouth of the lorica.

Möbius (1888) called the lorica chitinous, though he makes no mention of chemical tests to support his statement. Tests for chitin are difficult to make on such a small object. With picro-nigrosin and Mann's methyl blue eosin, I got positive results: the lorica stained blue in each case. Yet with iodine in potassium iodide followed by zinc chloride the result was negative. A negative result was also obtained with Millon's reagent, suggesting that the lorica is not protein.

Origin of the lorica. The lorica is laid down in the first place by the swarmer. This, as soon as it has found a suitable settling place, becomes inactive, appears to withdraw its membranelles and cilia, rounds off the pointed anterior end, and undergoes a peculiar process of vacuolation. Just before this there are streaming movements in the cytoplasm. Then the greenish granules with which it is well stocked pass to the periphery and large vacuoles appear throughout the body (Text-fig. 4, fig. 29). This leads to inflation and the ciliate becomes half as large again. By this means the limits of the new lorica are determined, for it must be larger than the animal it shelters and allow for growth. Complete immobility follows for about 5 minutes. Then there is a slight oscillating movement of the greenish granules. Meanwhile the vacuolation has increased, the lorica becomes very definite on the surface of the cytoplasm, and, 10 minutes or so after, the animal seems to be composed almost entirely of one enormous vacuole and appears dead. Only 5 minutes after this, vacuolation is reduced, movement of the cytoplasm recommences, and withdrawal from the lorica begins (Text-fig. 4, fig. 30). This starts in the region of the membranelles, which now reappear and, apparently better developed, become active. The contractile vacuole can now be seen again. Roughly an hour after the swarmer first becomes free it is established. That there is some close connexion between the granules and lorica-formation is obvious, but I am unable to say how the material in the granules reaches the surface and gets deposited there. In each case, however, some of the granules are left behind adhering to the newly formed lorica neck when the ciliate withdraws. Moreover, although normally rare in the peristome region, at this time they are numerous there.

Opening of the mouth of the lorica takes a variable time and I am unable to say whether it is accomplished by mechanical or chemical means. The ciliate moves backwards and forwards in the lorica, using its adoral membranelles. As it moves forwards, the proboscis creeps along the neck of the

lorica to which its dorsal surface is closely applied. When it gets to the closed end it bends over, still keeping in close contact. At this stage the ciliate seems to be pushing against the closed end with the back of its neck (Text-fig. 4, fig. 31). This lasts only a short time, and is followed by return to the base of the flask. Then the cycle of movements is repeated. Finally a break through is made and the ciliate can extend its proboscis and feed.

Additions to the lorica. Unlike the ciliates *Lagenophrys tatersalli* Willis (1942) and *Folliculina ampulla* Fauré-Fremiet (1932), where no additions are made to the adult lorica, *Chaetospira* adds to its from time to time. More frequent additions are made in the young than in the old ciliate. In all its stages the process of addition is similar to that of lorica formation by the swarmer, except that when inflation takes place the front end of the vacuolating *Chaetospira* emerges as a sphere from the mouth of the lorica (Text-fig. 4, fig. 32). The surface of this sphere is continuous with the neck of the lorica. After some time, during which presumably the surface of the sphere is converted into lorica material, the cytoplasm within the sphere reorganizes itself into the peristome of the ciliate. This now withdraws from the sphere which shrinks a little (Text-fig. 4, fig. 33). An opening is made at the distal pole of the sphere and it contributes a new length of neck to the lorica. Old specimens have very long necks. The rest of the surface of the ciliate contributes a new layer within the old lorica wall.

Encystment

Cysts occurred only in one batch of material, where they were discovered in encrustations at water-level in the winter of 1944–5. The first stages of encystment were not seen, nor was excystment.

Living cysts are pear-shaped and measure from 35 to 55 μ in length and 15 to 20 μ in breadth. Each lies in the lorica of the individual which forms it, and has a perfectly smooth wall 1 μ thick, except at the pointed end lying next the neck of the lorica. Here it thickens to 2 μ or more (Text-fig. 4, fig. 34). The contents are in close contact with the cyst all the way round and are finely granular and homogeneous, very different from the cytoplasm of the adult. There are no greenish granules, no cilia, no contractile vacuole or other organs.

The most common number of macronuclei in a cyst is three (Text-fig. 4, fig. 35), but cysts with two to six have been seen. This suggests that nuclear division might take place within the cyst as in *Gastrostyla* (Weyer, 1930) and *Kahlia simplex* (Horvath, 1936). One or two micronuclei are present. These I have not seen dividing. The nuclei have the same appearance as those of the unencysted adult.

No work has yet been done on the factors causing encystment. Since the pH of the medium has been maintained constantly at 7, this factor alone is not responsible. Since cysts were obtained only in winter months, drought and/or lowered temperature might be responsible. Cysts were not obtained the following winter (1945–6), so temperature alone is not the cause.

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I wish to express my sincere gratitude to Professor D. L. Mackinnon, who has given me constant help and encouragement.

SUMMARY

1. The structure, behaviour, and asexual reproduction of a plant-cell-inhabiting hypotrichous ciliate are described.

2. The systematic position of the ciliate is discussed and it is placed in the genus *Stichotricha* Perty as *S. intermedia* n.sp.

3. New information concerning behaviour, nuclear apparatus, and asexual reproduction is given for the loricate *Chaetospira mülleri* Lachmann. Cysts are described for the first time.

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Oogenesis in the Desert Snail *Eremina desertorum* with Special Reference to Vitellogenesis

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Contents

Introduction

Ancel (1903), working on *Helix pomatia*, was the first to give some attention to the study of oogenesis in Helicids. He described the history of the chromatin, the nucleoli, and the cytoplasmic bodies. In his account of the chromatin cycle, however, this author overlooked the important stages of chromosomal conjugation and simply detailed the stages of chromatin diffusion. Ancel's account of the history of the cytoplasmic bodies is very defective owing to the crude technical methods used in cytoplasmic cytology in his time.

Gatenby (1917), in a work primarily focused on the cytoplasmic inclusions of germ cells of *Helix aspersa*, gave subsidiary attention to the chromatin cycle in oogenesis. In fact he did not recognize any of the meiotic prophases except the pachytene 'bouquet'.

Making use of his Flemming without acetic and iron haematoxylin technique, Gatenby could demonstrate and follow satisfactorily the history of mitochondria in oogenesis. He also detected in the ooplasm certain 'Neberkern' bodies (Golgi bodies), but he did not follow their history in any detail. This author could also see in an homogeneous juxtanuclear zone of the ooplasm several blocks of darkly staining material. 'The nature of these structures (Gatenby states) and their connexion if any with mitochondria or Neberkern is unknown to me' (loc. cit., p. 589).

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In 1920 Gatenby together with Woodger reinvestigated oogenesis in *Helix* and also studied the same process in *Limnaea* and *Patella*, with the aim of disclosing the mechanism of yolk formation. They observed that especially in *Patella*, the Golgi bodies were plastered on the yolk spheres. This association was taken by the above authors as evidence of the direct metamorphosis of Golgi rods into yolk. Concerning mitochondria, they note that though these elements show growth activities during deutoplasmogenesis, especially in *Limnaea*, much of the evidence is against the view that part of the mitochondrial constituents of the cytoplasm metamorphose into yolk.

Ludford (1921) repeated Gatenby and Woodger's work on *Patella*. He noted that the primordia of the deutoplasmic spheres are deposited under the influence of Golgi bodies and not (as his predecessors thought) that the Golgi bodies change into yolk. Mitochondria, in Ludford's belief, have no direct concern with vitellogenesis.

Brambell (1924) described in the oocytes of *Helix* and *Patella* two distinct categories of deutoplasmic reserve products. He preferred to designate each category according to the cytoplasmic body concerned in its production. Thus he distinguished 'Golgi yolk' and 'mitochondrial yolk'. In the case of *Patella*, he confirmed Ludford's view that the Golgi yolk is formed by, not from, the Golgi rods. In both *Patella* and *Helix*, according to this author, 'mitochondrial yolk' arises through the swelling of mitochondria. Writing about the nature of the Golgi yolk, he favoured the view that it is essentially fatty in both *Patella* and *Helix*.

From the above survey it becomes evident that our knowledge of oogenesis in Pulmonates is still immature. In so far as the chromatin cycle is concerned, it is hardly necessary to say that up till now the full sequence of the Helicid oocytic prophases still abides in the dark and calls for consideration.

Information about the behaviour and role of the two categories of cytoplasmic bodies, viz. Golgi bodies and mitochondria, is more plentiful, but unfortunately contradictory, especially in relation to the part each plays in vitellogenesis. Workers are not agreed as to the exact process by which a Golgi element gives a deutoplasmic sphere. Some consider the process a direct metamorphosis of the one element into the other. Others think that yolk is formed by, not from, the Golgi elements. Also, no agreement is reached as to whether or not mitochondria take a direct part in deutoplasmogenesis. Such a difference of opinion regarding most of the essential points of the mechanism of yolk formation made further study of oogenesis in Pulmonates urgently desirable. Accordingly, Professor K. Mansour (Dept. of Zoology, Fouad I University, Cairo) suggested that the author should undertake a study of the oogenesis in the desert snail *Eremina desertorum*.

Material and Technique

The desert snail *Eremina desertorum* is a pulmonate Mollusc which occurs in fair abundance in the Egyptian deserts. The material for the present study was brought from Abu-Rawash, a desert district near Cairo.

The desert snail seems to respond readily to environmental conditions. Early in summer (sometimes even late in spring) the animal enters its aestivation period which continues till autumn. Copulation takes place during January. The egg-laying period extends from the middle of February till about the end of March.

As in all Helicids, the hermaphrodite gland of the desert snail lies embedded in the tissue of the liver, at the apex of the visceral hump. The ovotestis was quickly dissected out of the surrounding liver tissue, excised, cut in three or four pieces, and immediately plunged into the fixative.

Most suitable fixation of the nuclear structures was attained by using strong Flemming's mixture for 24 hours. Ordinary Bouin's fluid or urea/Bouin combinations (Ezra Allen's, or Eleanor Carother's; see *Vade Mecum*, 9th edition, p. 319) were also found to be suitable. The nuclear stains used for revealing the chromosomes were iron-haematoxylin and gentian-violet iodine.

For detecting chromatin (strictly speaking the thymonucleic acid thereof), nothing was found more reliable than Wermel's (1927) modification of Feulgen's 'Nuclealfarbung' method.

For the determination of the chromophil nature of the nucleoli, Scott's Ehrlich haematoxylin and Biebrich scarlet method proved unsurpassed. Karyosomes took the haematoxylin, and plasmosomes the orange-red colour of Biebrich scarlet. Recourse was also made to Mann's methyl blue/eosin; Bensley-Cowdry methyl green acid fuchsin and Pappenheim's methyl green/pyronin techniques. Gatenby's toluidine blue/eosin method was also of great use.

For the demonstration of mitochondria, chromo-osmium fixatives F.W.A., Champy, and Nassonov gave admirable results. Formalin-chrome fixatives of Regaud and Bensley-Cowdry were not very successful with the desert snail's ovotestis; they caused granulation of the mitochondria. The best stain combinations for revealing the mitochondrial material of the desert snail's germ cells were found to be iron haematoxylin/orange G, iron haematoxylin/erythrosin, Altmann's acid fuchsin, and Champy-Kull's acid fuschsin/toluidine blue/aurantia.

The Golgi bodies of the germ cells of the desert snail are preserved by the mitochondrial fixatives, but become most pronounced after post-osmication. Nassonov's modification of Kolatchev's osmium-impregnation technique was by far the best. The Mann-Kopsch and Kopsch's methods also gave good results. The silver-impregnation methods of Da Fano and Cajal, though successful, caused a good deal of shrinkage. Post-osmicated material was either left unstained or stained in Ludford's neutral red.

The demonstration of the Golgi bodies and mitochondria in one and the same cell was found to be an easy matter in all stages of oogenesis of the desert snail. Pieces of the ovotestis were impregnated by Nassonov's technique for 4 days in an incubator at 37° C. This time was just enough for the impregnation of the Golgi bodies, while the mitochondria remained unchanged. Sectioned material was subsequently stained in Altmann's acid fuchsin and

then in aurantia. The Golgi bodies and fat appeared black, the mitochondria red, and the ground cytoplasm golden yellow.

For the detection of fat, Nath's (1934) technique with Sudan III and Scharlach R was tried on fresh and formalin-fixed material and gave good results.

The modern techniques of Baker (1944 and 1946) for the detection of some of the intracellular inclusions by the application of proper chemical procedures on frozen sections of fresh or formalin-fixed material were extremely helpful. His method for the recognition of lipin (acid haematein in conjunction with pyridine extraction) was applied on grown oocytes and enabled the differentiation between mitochondria and proteid yolk.

Lastly, the study of centrifuged oocytes yielded very interesting results.

Histological Features of the Ovotestis

The ovotestis of the desert snail is constituted of elongated branched diverticula (Text-fig. 1), opening into the hermaphrodite duct. Although the lumina of some terminal diverticula rarely appear slightly distended, there are no typical globular acini. The ovotestis of the desert snail is therefore more nearly digitate than acinous.

The wall of each diverticulum consists of an outermost layer of connective tissue internal to which comes the germinal epithelium. Each diverticulum during the full swing of germ-cell production contains male, female, and nurse cells (Text-figs. 1 and 2). The female cells and the nurse cells appear close to the wall of the diverticulum whereas the male cells fill its lumen. The earlier stages of the male germ line (the spermatogonia and the spermatocytes) are found nearer to the wall of the diverticulum, while the later stages (the spermatids and the spermatozoa) are found towards the centre of the diverticulum. It must be noted, however, that the concentric arrangement of the successive stages of the male germ line is by no means clear cut and decisive. Sometimes one finds a few spermatocytes lying in the centre of the diverticulum, or a few sperms near the periphery. Towards the blind end of the diverticulum one finds a greater abundance of the earlier male germ cells (Text-figs. 1 and 3), whereas at the open end there appears a greater number of the later stages of the male germ line (Text-fig. 2).

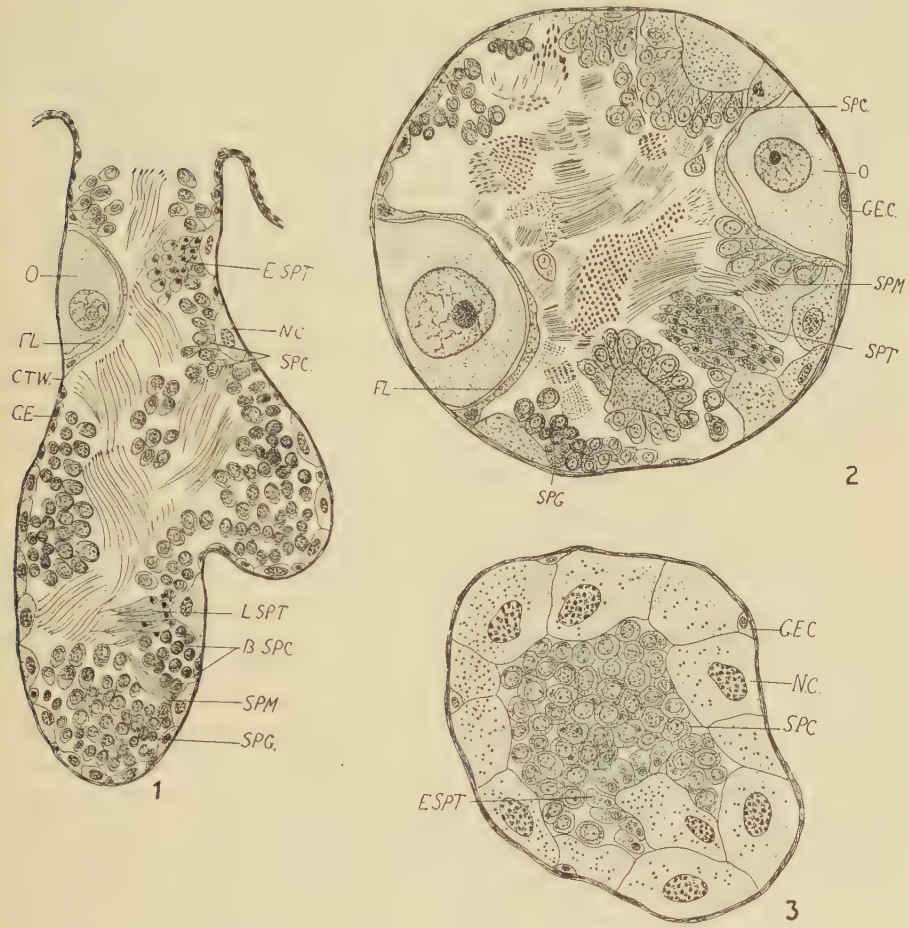
Oocytes may be seen anywhere on the wall of the ovotestis diverticulum, but most frequently nearer to its mouth (Text-figs. 1 and 2). These female elements invariably begin their development underneath a layer of nurse cells and thus remain separated from the male germ cells throughout the whole of their history. A fully differentiated oocyte appears surrounded by a follicle of nurse cells (Text-fig. 2). This follicle gradually dwindles with the progress of oocytic growth and finally disappears when the oocyte is about to pass to the hermaphrodite duct.

Nurse cells are by no means restricted to the oocytic area of the ovotestis diverticulum. They are most numerous and hypertrophied towards the blind ends of the diverticula, where the early stages of the male germ line occur in abundance (Text-fig. 3).

The Germinal Epithelium and its Differentiation

Cytological Structure of the Germinal Epithelium

In its undifferentiated condition, the germinal epithelium is constituted of a continuous layer of flattened cells with oval, flattened nuclei (Text-fig. 4).



Text-figs. 1–3, Sections of the ovotestis diverticula.

Fig. 1, L.S, from a Bouin preparation; × 180. Fig. 2, T.S, near open end, and Fig. 3, T.S, near blind end, both from F.W.A. preparations; × 240.

B.SPC, bouquet spermatocyte; C.T.W, connective tissue wall; E.SPT, early spermatid; FL., follicle; G.E, germinal epithelium; G.E.C, germinal epithelial cell; L.SPT, late spermatid; N.C, nurse cell; O, oocyte; SPC, spermatocyte; SPG, spermatogonium; SPM, sperm; SPT, spermatid.

The chromatin of the nucleus of the germinal epithelial cell is in the form of blocks of irregular shape and unequal size (Text-fig. 4). The number of these blocks is high and also subject to marked variation.

In spite of using a great diversity of fixatives, it was impossible to detect

any connecting bridges between the chromatin blocks of the germinal epithelial nuclei as was described by Gatenby (1917) in the corresponding cells of the garden snail. Ancel (1903) did not describe any such connectives between the chromatin blocks of the germinal epithelial nucleus of *Helix pomatia*. In this respect, therefore, the chromatin of the germinal epithelium of the desert snail is similar to the corresponding chromatin of *H. pomatia*.

The chromatin of the germinal epithelial nucleus takes the basic dyes much better than that of any other cell of the male or female line. Applying Wermel's modification of Feulgen's reagent on sections of the ovotestis of the desert snail, the chromatin blocks of the germinal epithelial nuclei, as well as those of the nurse-cell nuclei, showed the deepest violet colour. The thymonucleic acid content is therefore maximal in the undifferentiated germinal and nurse-cell nuclei.

Nucleoli are absent from the germinal epithelial nuclei. This was ascertained by the application of Wermel's reaction followed by light green. No green colour appeared.

The cytoplasm is not abundant in the germinal epithelial cells. In the majority of cases the nucleus occupies almost two-thirds of the volume of the cell, the cytoplasm the remaining third. Fixing in F.W.A. (diluted by one-third its volume distilled water) and staining in iron haematoxylin and erythrosin, the cytoplasm took the form of an homogeneous reddish mass.

The mitochondrial material of the germinal epithelial cell was also demonstrated by the above F.W.A./iron haematoxylin technique. To one side of the nucleus it was possible to detect a zone consisting of several fine, deep-black, mitochondrial granules embedded in a darkly staining matrix of ground cytoplasm. Also, in the paranuclear zone and rarely in other places of the cell, a few bigger mitochondrial granules may be met with in some, but not all, the cells (Text-fig. 5).

The Golgi bodies of the germinal epithelial cell were not always successfully demonstrated by the F.W.A./iron haematoxylin technique. In a few cases, however, when fixation was prolonged to 3 or 4 days and the stain also prolonged to 24 hours in each of the baths of iron alum and haematoxylin, it was possible to detect in a few germinal epithelial cells black bodies bigger in size than mitochondria, recalling the Golgi bodies (Text-fig. 5). On fixing the ovotestis in Nassonov (chrome-osmium-dichromate) and treating with 2 per cent. osmic for 5–7 days in an incubator at 37° C., the Golgi bodies appeared clearly as deep-black curved rods from 6 to 8 in number situated near one pole of the nucleus (Text-fig. 6). Staining the post-osmicated sections with Altmann's acid fuchsin revealed the mitochondrial cloud as a reddish zone around the Golgi bodies, thus proving that the Golgi bodies and the mitochondrial cap are present at one and the same pole of the nucleus.

Differentiation of the Germinal Epithelium

The period of germ-cell differentiation in the desert snail seems to begin by the end of the aestivation period and continues till late spring or early

summer of the following year. At the beginning of this period the germinal epithelium gives rise to the male elements. These elements separate from the wall of the diverticulum and fall into the lumen where they multiply. Almost simultaneously, some of the germinal epithelial cells give rise to nurse cells which gradually grow and spread over the so far undifferentiated germinal epithelial cells. Eventually the wall of the ovotestis diverticulum appears organized into two layers, an inner layer of nurse cells and a peripheral layer of undifferentiated germinal epithelial cells. The cells of the peripheral layer do not differentiate until about the middle of the reproductive cycle, and when they do, they give rise to the female elements.

The earliest symptoms of differentiation in the germinal epithelial cells are the same irrespective of whether male or female elements will ultimately result. In the nucleus, the chromatin blocks which were numerous and small in size in the germinal epithelial nucleus (Text-fig. 4) seem to aggregate together into fewer, more voluminous chromatin masses. Such chromatin masses are no longer completely separate from one another, but are seen to be connected here and there by short, faintly coloured chromatin filaments (Text-fig. 7). Inside the nucleus and a bit to one side one generally finds a nucleolus which takes basic dyes but faintly. Its complete freedom from thymonucleic acid is proved by the negative reaction it gives with Wermel's reagent.

The paranuclear zone of mitochondrial material, already indicated to be present in the cytoplasm of the germinal epithelial cell, becomes in the early germ cell markedly bigger and contains a greater number of big mitochondrial granules (Text-fig. 8).

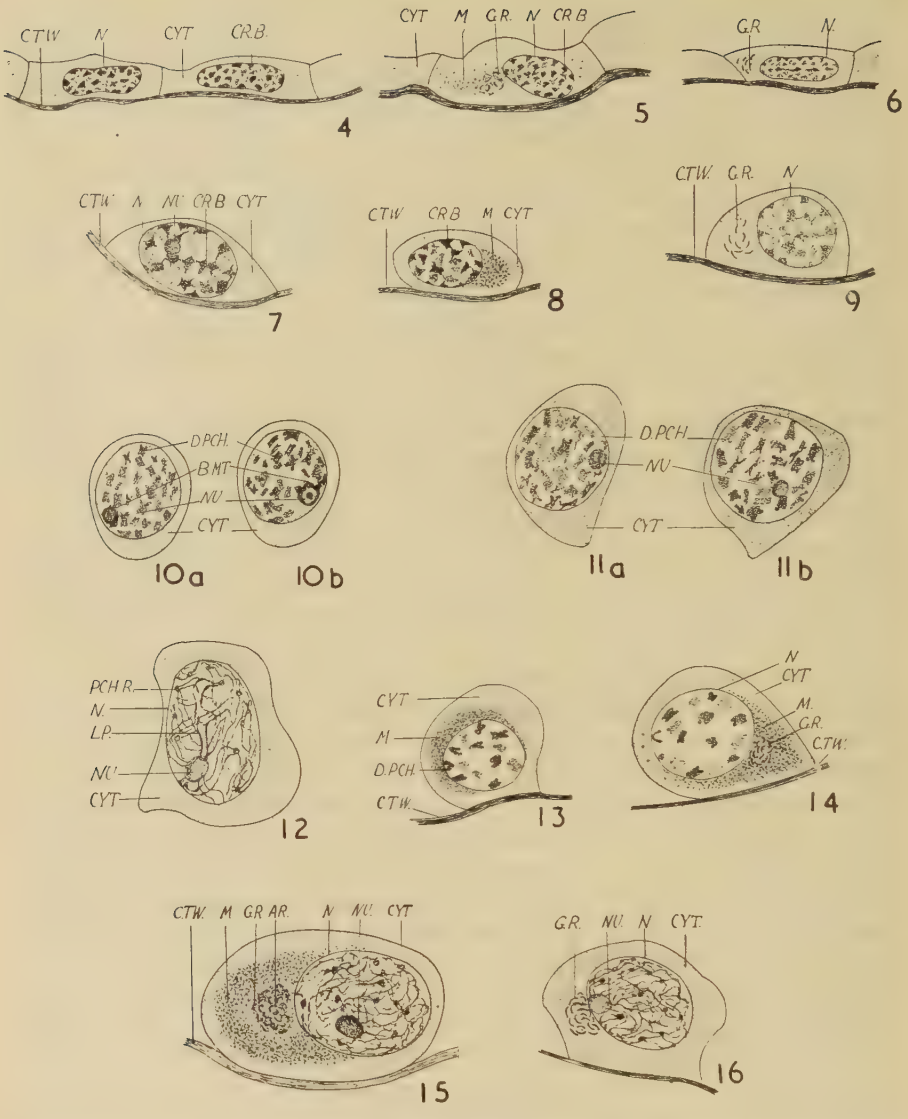
The Golgi bodies of these early germ cells, though still not easily demonstrable by the F.W.A./iron haematoxylin technique, can be brought up marvellously by Nassonov's post-osmication method. As in the case of the germinal epithelium, the Golgi bodies appear as curved osmiophil rods, differing only in being longer and finer. Moreover, these bodies are no longer close to one another but appear dispersed in a bigger zone (Text-fig. 9).

It is from the above-described stage that both male and female lines begin to develop. The name 'Cellule progerminative indifferent', put forward by Ancel (1903) to the germ cell at such a stage, seems most favourable.

In this paper the details of the differentiation of the female elements from the progerminative indifferent cells will be given. The mode of differentiation of the male and nurse cells will be described elsewhere.

The Earliest Female Elements

Nuclear behaviour during the differentiation of the female elements is very characteristic. The chromatin of the progerminative indifferent cell becomes resolved into a few big masses of variable sizes (Text-figs. 10 and 11). Apart from these chromatin masses there exists in the nucleus a voluminous nucleolar formation, which appears constituted of a central sphere out of which extend a few irregular bodies (Text-fig. 10). The central sphere



TEXT-FIGS. 4–16. Differentiation of the germinal epithelial cells into the earliest oocytes.

Figs. 4–6, undifferentiated germinal epithelial cells, Figs. 7–9, progerminative indifferent cells, Figs. 10–16 earliest oocytes. Figs. 4, 7, 10, 11, and 12 from Flemming with acetic, Figs. 5, 8, 13, 14, and 15 from F.W.A., and Figs. 6, 9, 16 from Narsonov's post-osmication preparations. All figs. × 1360.

AR, archoplasm; B.MT, basiphil material, CR.B, chromatin body; CYT, cytoplasm; D.PCH, double prochromosome; G.R, Golgi rod; LP, leptotene chromosome; M, mitochondria; N, nucleus; NU, nucleolus; PCH.R, prochromosome remnant. Other lettering as before.

consists of a strongly basiphil core and a weakly basiphil rim. The irregular bodies surrounding it are strongly basiphil. Soon afterwards, the irregular bodies disappear and we are left with the central sphere; now staining weakly and homogeneously in basic dyes and forming the earliest oocytic nucleolus (Text-fig. 11).

Focusing the attention now on the chromatin masses (Text-fig. 11), it can be observed that each has the form of a slightly elongated, faintly staining, dual body. Evidence of duality is denoted by the presence of a cleft at both of its extremities. In rare cases that cleft is seen extending along the whole length of the element.

Repeated counts revealed that the number of the dual chromatin masses is 28. The diploid chromosome number of the desert snail, as counted from spermatogonial mitotic plates, is 56. The masses under consideration correspond, therefore, to the haploid number. Being dual, they represent the diploid complement of the female element and, therefore, one is justified at this stage to refer to them as 'double prochromosomes'.

The double prochromosome stage soon gives rise to the unravelling stage. Each double prochromosome becomes resolved into two fine chromatin threads and a spherical remnant. The threads are the early leptotene threads; the remnant on the other hand gives the same colour reactions as the nucleolus and is therefore of the same nature (Text-fig. 12).

While these processes are taking place inside the nucleus, the whole element is increasing in size; the cytoplasm more than the nucleus. The growth period of the female element, therefore, begins the moment it is cytologically detectable. Concurrently, certain important changes take place in the cytoplasmic inclusions.

The mitochondria in the earliest female element constitute a well-defined cap closely applied to one side of the nucleus (Text-fig. 13). This cap appears in F.W.A./iron haematoxylin preparations as a darkly staining cytoplasmic ground matrix containing several jet-black, fine, and slightly coarser mitochondrial granules. Later the mitochondrial cap grows quickly in size and becomes more stainable. Its shape becomes more or less cone like; the point of the cone directed towards the cell periphery, the base closely abutting on to the nuclear membrane (Text-fig. 14). This cone now contains numerous large mitochondrial granules.

In a section passing through a larger oocyte still at the leptotene stage (Text-fig. 15) the mitochondria appear as a huge U-shaped formation, with the end of the U directed towards the nucleus and the base towards the cell periphery. The mitochondrial zone now appears constituted of a tremendous number of well-defined granules which still appear embedded as before in a darkly staining cytoplasmic ground matrix. The latter matrix probably contains mitochondrial material in colloidal dispersion since it does not appear except after mitochondrial techniques.

The Golgi bodies in the youngest oocyte, at the double prochromosome stage, are represented by several slightly curved rods lying nearest to the

nuclear membrane in the zone of the maximal mitochondrial aggregation (Text-fig. 14). About the time the prochromosomes have given rise to the early leptotene filaments, the Golgi bodies appear in F.W.A./iron haematoxylin preparations as curved C-shaped bodies aggregated around a mass of archoplasm (Text-fig. 15). After the Nassonov post-osmication method, the Golgi rods appear as numerous slightly curved rods closely aggregated together; but the archoplasmic mass was not impregnated (Text-fig. 16).

The Growth Period of the Oocyte and Yolk Deposition

In the previous section the female element was left when it was easily distinguishable as such, namely when its nucleus reached the leptotene stage. During the growth of this early element to the fully formed oocyte, both the nucleus and the cytoplasmic inclusions undergo definite changes. A detailed description of these changes is given below.

The Chromatin and Nucleoli

The juxtaposed chromatin threads of the leptotene nucleus (Text-fig. 12) soon appose side by side and then quickly wind round one another to give the strepsitene double spirals (Text-fig. 17). Very rarely does one find a post-leptotene nucleus with all its pairs of synaptic mates apposed without being relationally coiled. It seems, therefore, that the pachytene stage in the desert snail's oocyte is of very short duration and quickly leads to the strepsitene one. The pachytene, as well as the strepsitene elements of the oocyte, are always disposed more or less radially in the oocytic nucleus, polarized towards a central nucleolus.

In a later oocyte (Text-fig. 18), each strepsitene double spiral shortens, condenses, and becomes more basiphil; now identifiable as a diplotene bivalent. The bivalents are arranged peripherally just underneath the nuclear membrane. Repeated counts from thick sections made it possible to ascertain the haploid number of bivalents, viz. 28.

After diplotene, the oocyte grows a little further and the chromatin begins to enter the diffusion or dispersion, characteristic of a typical germinal vesicle. The bivalents become attenuated and lose almost completely their basiphily (Text-fig. 19). Later the chromatin threads appear as linear series of fine granules (Text-fig. 20). However, until now the identity of the diplotene elements, and sometimes even their duality, is vaguely manifest.

With the progress of growth, the chromatin threads become clumped into a few stellate or irregular formations (Text-fig. 21). Now the identity of the diplotene elements is almost totally masked.

Towards the end of growth, the dispersion of the chromatin reaches its maximum. Now one can see only several chromatin tracts, loose in texture and outline, either free from one another (Text-fig. 22) or partially intercommunicating (Text-fig. 23). Where they are thoroughly free, one can notice that they seem as if suspended in a framework (Text-fig. 22). In the author's

belief, this framework resulted from the coagulation of the enchylema of the germinal vesicle.

As previously indicated, an oocyte at the leptotene stage (Text-fig. 12) possesses a large nucleolus and several small prochromosome remnants which are, in fact, nucleolar in nature.

At the pachytene and strepsitene stages, from two to four nucleoli of fairly large size occur. In Text-fig. 17 three nucleoli are shown; one acting as the polarization centre. Comparing Text-figs. 12 and 17, it becomes evident that some of the material of the nucleoli in the latter oocyte was derived in all probability from the prochromosome remnants.

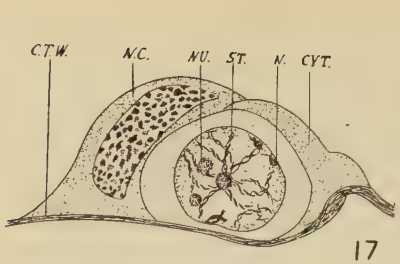
The staining reactions of the nucleoli at the leptotene as well as at the pachytene and the strepsitene stages point to a slight basiphily. However, with Wermel's thymonucleic acid test, the nucleoli do not show the slightest violet tinge. They are, therefore, in spite of their slight basiphil stainability, typical plasmosomes.

During diplotene and early diffusion, nucleoli gradually fuse to give a large single nucleolus. In Text-fig. 18 there occurs the principal nucleolus and a small accessory one. Soon after (Text-figs. 19 and 20), this too fuses with the growing principal nucleolus.

Later in diffusion, when the chromatin of the germinal vesicle appears in the form of stellate formations, there occur, in addition to the large old nucleolus, other very small nucleoli in formation (Text-fig. 21; E.NU). In F.W.A./toluidine blue/eosin preparations the newly developing nucleoli can be identified in the midst of the stellate, now oxyphil (reddish), chromatin clumps, as small spherical bodies which, like the principal nucleolus, stain bluish-red. In the same nucleus one can identify various sizes of such nucleoli ranging from large granules to small spheres. As growth of the oocyte progresses, the newly formed nucleoli gradually fuse with the old principal nucleolus. In the germinal vesicle of the oocyte depicted in Text-fig. 22 there occurs, apart from the principal nucleolus, a much smaller accessory one, closely associated with it. This, too, is destined to fuse with the principal nucleolus, so that towards the end of the growth period we are left with a single giant nucleolus towards the middle of the germinal vesicle (Text-fig. 23).

The moment the oocyte's principal nucleolus reaches a fairly large size, at or shortly before chromatin diffusion, its stainability changes. Originally taking weakly and homogeneously the basic dyes, it now stains heterogeneously; certain parts stain more acidophil, others more basiphil. In Text-fig. 18 the acidophil part (NU.A) is rounded up excentrically in the nucleolus, and the basiphil part (NU.B) constitutes a peripheral crescentic zone. In Text-fig. 20 there can be seen in the midst of the nucleolus two adjacent acidophil zones, one spherical, the other bean-shaped; the rest of the nucleolus is basiphil.

In a fairly grown oocyte (Text-fig. 22), the major part of the nucleolus appears acidophil, the basiphil material being contained in a small zone to one side. Also, just underneath the nuclear membrane a few small nucleolar



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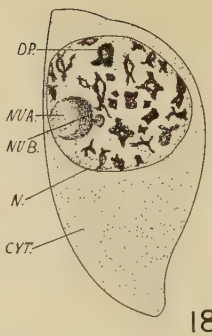

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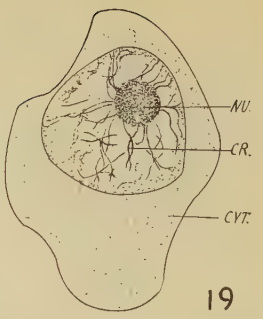

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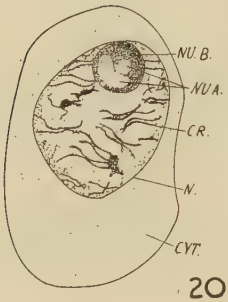

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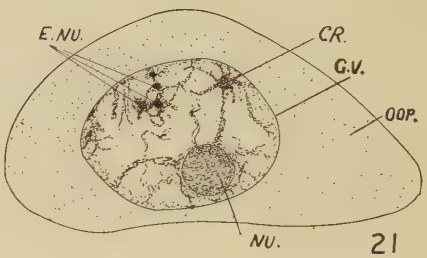

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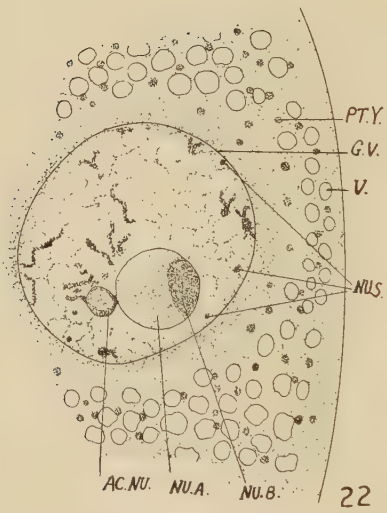

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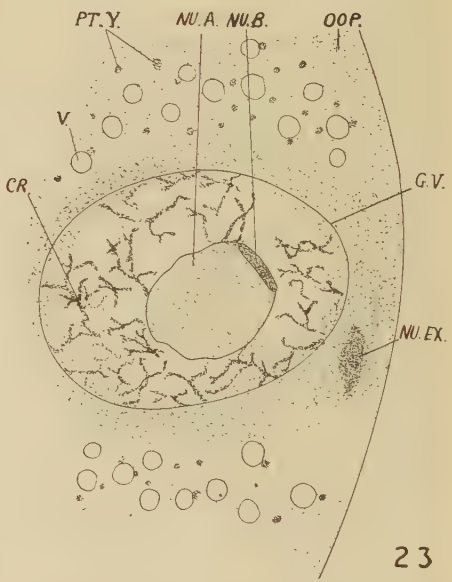

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TEXT-FIGS. 17–23. Behaviour of the chromosomes and the nucleoli in the growing oocytes.

Fig. 17, strepsitene stage; Fig. 18, diplotene stage; Figs. 19–23, successive stages in the denucleination of the chromosomes during the formations of the oocytic germinal vesicle. All figures are from Flemming with acetic/Scott preparations. Figs. 17–21 × 1360; Figs. 22 and 23 × 680.

AC.NU, Accessory nucleolus; CR, chromatin; E.NU, early nucleoli; G.V, germinal vesicle; N.M, nuclear membrane; NU.A, acidophil part of nucleolus; NU.B, basiphil part of nucleolus; NU.EX, nucleolar extrusions; NU.S, nucleolar spherules; OOP, ooplasm; P.NU, principal nucleolus; PT.Y, proteid yolk; ST, strepsitene bivalent; V, vacuole. Other lettering as before.

spherules staining basiphil can be detected (NU.S). Soon afterwards the nucleolar basiphil material markedly decreases; now the whole nucleolus is acidophil, except for a very small basiphil part extending to one side (Text-fig. 23), NU.B.). Also the small basiphil nucleolar spherules are no longer apparent. At the same time, a mass of material, staining just like the basiphil part of the nucleolus, appears outside the nuclear membrane in the ooplasm.

Although no granular emission has been observed either from the nucleolus into the nucleoplasm, or from the nucleus into the cytoplasm, yet the coincidence of the appearance of the ooplasmic mass with the decrease in the basiphil nucleolar material seems to furnish inferential evidence for some type of nucleolar extrusion. The extrusion, most likely, is of material in solution, as was tentatively suggested by Harvey for Ciona (1927), Carcinus (1929), and Antedon and Asterias (1931). The probability is that the nucleolar basiphil material transformed into a liquid phase or rendered in solution, passes as such through the nuclear membrane and once in the ooplasm recollects again to construct formed bodies. The small nucleolar spherules, observed just to the interior of the membrane of the germinal vesicle in some oocytes, may have resulted from the coagulation of the emitted nucleolar material before its final exit into the ooplasm.

Similar ooplasmic basiphil masses were observed and described by Gatenby (1917) in the oocytes of the *Helix aspersa*. However, he did not discuss either their origin or their nature.

The Cytoplasmic Inclusions and Deutoplasmic Bodies

In the early oocytes we have already seen that the mitochondrial zone consisted of a number of granular mitochondria embedded in a darkly staining ground matrix. At about the leptotene stage the same zone appeared as a fairly large U-shaped formation.

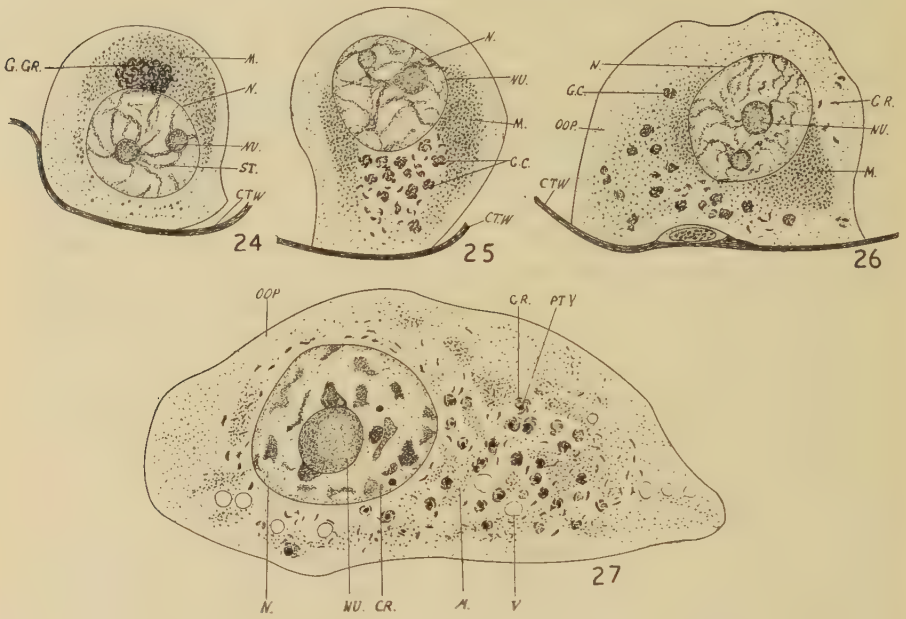
Later, at the pachy-strepsitene stage (Text-fig. 24), the mitochondrial zone increases still more in size and now extends from the nuclear membrane to very near to the cell periphery. The mitochondrial granules themselves are larger in size and darker in stainability than before. Away from the mitochondrial zone a few separate mitochondrial granules may appear around the nucleus. Soon after (Text-fig. 25), the mitochondrial zone seems to be divided into two subequal groups.

As the oocytic growth becomes pronounced, the mitochondrial granules quickly increase in number and begin to disperse in the ooplasm. Text-fig. 26 represents an early stage in mitochondrial dispersion. Here again two mitochondrial groups are detectable; in one group the close aggregation of the mitochondrial granules is still preserved, in the second, mitochondria have partially dispersed. The mitochondrial granules in the dispersion zone are slightly finer than those in the other zone where dispersion is not pronounced.

As the oocyte reaches a fairly good size (Text-fig. 27), just at the beginning of yolk deposition, mitochondria become dispersed somewhat unevenly in the

cytoplasm. Groups of fine mitochondrial granules are met with here and there in the oocyte's cytoplasm.

Towards the end of growth (Text-figs. 33 and 34), when yolk deposition has well progressed, mitochondria appear evenly distributed all over the ooplasm. Now most of the mitochondrial granules are undoubtedly larger than those in the earlier oocytes of the stage shown in Text-fig. 27, but they are still much smaller than the smallest yolk spheres.



TEXT-FIGS. 24–27. Mitochondria, Golgi bodies, and yolk in oocytes at the early stages of their growth.
All figs. from F.W.A. preparations; × 1360.
G.C, Golgi complex; G.GR, localized Golgi group. Other lettering as before.

It was previously indicated that the oocyte's Golgi elements at the leptotene stage appeared as a group of osmiophil slightly curved rods (Text-figs. 15 and 16). At the stages immediately following (pachytene and strepsitene), the number of the Golgi rods appears to be greater and their size larger (Text-figs. 24 and 28). Still they lie aggregated in a group to one side of the nucleus.

As the oocyte grows, the Golgi group also enlarges, both by enlargement of the individual rods and by the appearance of more smaller rods presumably through fragmentation of the older rods. By the stage depicted in Text-fig. 29, the Golgi juxtanuclear group appears large, and also several rods appear to have migrated into the cytoplasm from the main group.

In slightly older oocytes (Text-fig. 30) the Golgi elements appear widely distributed almost all over the ooplasm. Some of the scattered Golgi rods lie singly in the cytoplasm, but others are grouped to form complexes very

characteristic of the oocyte of the desert snail at, and immediately after, the stage at issue (Text-figs. 30 and 31).

In F.W.A./iron haematoxylin preparations, the Golgi bodies were usually detected (Text-figs. 24, 25, 26, 27). The appearance of the oocytic Golgi complex after this technique is typical. Each complex consists of an archoplasmic mass with the Golgi rods arranged on its perimeter. The curved Golgi rods are disposed with their concave sides facing centrally, i.e. towards the archoplasm.

After the scattering of the Golgi bodies has fairly proceeded, yolk deposition sets in. From the beginning, this deposition takes place in close association with the Golgi bodies. In the interior of the Golgi complexes, or on the concave sides of the separate Golgi rods, there appear minute yolk spherules (Text-figs. 27 and 31). While still in association with the Golgi rods the yolk granules seem to enlarge, because within different Golgi complexes granules of various sizes were seen (Text-fig. 31).

At this early period of yolk deposition, the ooplasmic masses referred to before are very prominent and occupy a juxtanuclear position (Text-fig. 31, NU.EX).

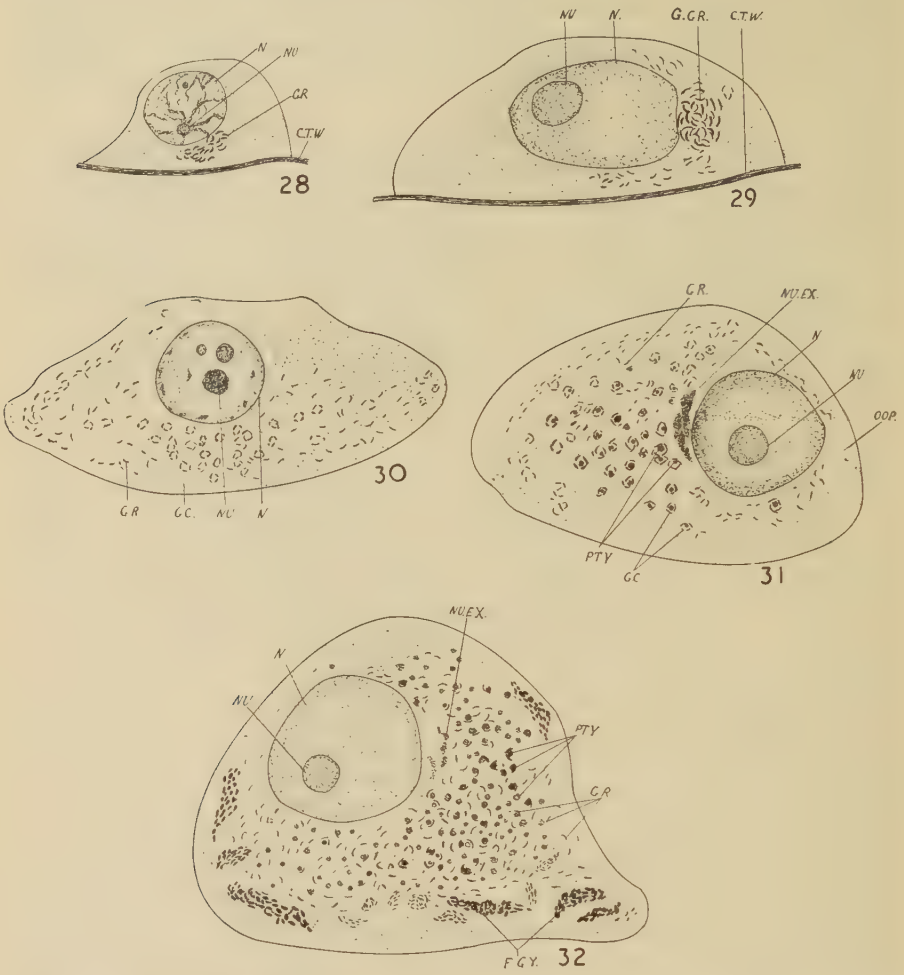
As vitellogenesis proceeds still farther, the cytoplasmic inclusions of the oocyte appear as shown in Text-fig. 32. Most of the Golgi complexes have now separated into their constituent rods. Rarely, some rods still appear associated in twos to form V-shaped formations. While some yolk spherules still appear associated with the Golgi bodies, others have migrated off into the ooplasm. Every step in this migration can be traced. The yolk spherule is first severed from the Golgi rod, then appears a short distance from it, and at last it is completely separate.

The ooplasmic masses are still manifest at this stage, but are undoubtedly much smaller than before (compare Text-fig. 31 with Text-fig. 32). Later, these masses are thoroughly lost to view, their material probably contributing to the raw material used in yolk formation.

After the Golgi bodies are severed from the yolk granules, they soon run together and form clumps of variable sizes scattered here and there in the ooplasm, especially towards the periphery (Text-figs. 32 and 33). In the early phases of the process, the rods of the clumps do not differ much from the Golgi rods of previous stages, except for a slight increase in osmiophily (the small clumps in Text-fig. 32). Later the rods increase in girth and thus appear almost oval (Text-fig. 33). Also they become much easier to impregnate with osmium, appearing black in chrome-osmium material even without post-osmication. Further, applying Sudan III and Scharlach R on fresh and formalin-fixed material, it was found that the oval elements of the clumps gave a positive test; these elements, therefore, must contain fat.

Most helpful and clarifying is the following test: post-osmicated sections were mounted in turpentine, a cover slipped over, and the extraction of osmium from the elements of the clumps was observed under the microscope. Gradually the girth of the elements decreased, till after a lapse of half an

hour their girth fell to its state before the clumping stage, being now of just the same girth as the unchanged Golgi rods of the early oocytes in the same section. Up till the end of the first hour no further appreciable extraction was



TEXT-FIGS. 28–32. The Golgi bodies before and during vitellogenesis in the growing oocytes.

All figs. from Nassonov post-osmication preparations. Figs. 28–30, ×1360; Figs. 31 and 32, ×680.

F.G.Y, fatty Golgi yolk. Other lettering as before.

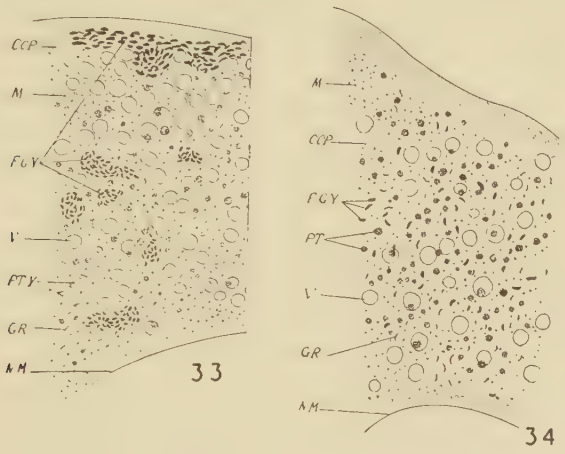
noticed. The slide was then uncovered and transferred to a jar of turpentine. Even after 3 hours neither the residual rods of the clumps in grown oocytes, nor those of the young oocytes, were decolorized.

On rare occasions, it must be noted, the Golgi bodies did not clump together in groups, but remained widely scattered in the ooplasm (Text-fig. 34).

However, in these cases also the increase in the girth of rods due to their loading with fat was plainly evident.

The natural conclusion, therefore, is that in the late growth period of the oocyte, some of the Golgi rods become loaded with a certain fat. The original Golgi element in the composite body, however, always preserves its identity and does not transform itself into fat.

The cytoplasm of the full-grown oocyte of the desert snail contains a fairly large number of vacuoles. These vacuoles appear in abundance fairly late in



Text-figs. 33 and 34. Portions of the ooplasm towards the end of growth. From Nassonov/Altmann's fuchsin/aurantia preparations; × 1020, lettering as before.

the oocytic growth, though a few may appear in earlier stages. In the fairly young oocyte depicted in Text-fig. 27, a few vacuoles were seen. The content of these vacuoles is most probably watery, since it gives negative reactions with all the fixing reagents used.

Between the vacuoles of the oocyte are embedded the four elements: yolk spheres, mitochondria, and the normal and fat-loaded Golgi bodies (Text-figs. 33 and 34). In some instances, yolk spheres are seen in the interior of the vacuoles. This may be attributed to the mechanical dislocations during preparation, since in early oocytes (Text-fig. 27) the first-appearing vacuoles did not envelop yolk spheres.

Even fairly late in growth, the distinction between the different cytoplasmic inclusions of the oocyte of the desert snail is by no means a difficult matter. In F.W.A./iron haematoxylin/erythrosin preparations the fat-loaded Golgi bodies appear as black oval grains; the normal Golgi bodies as fine curved blackish rodlets; the mitochondria as black granules of various sizes; the yolk spheres take the plasma stain faintly and thus appear yellowish-red. Most helpful, also, is the Nassonov/Altmann combination technique. After this method the mitochondria stain deep red, the fat-loaded Golgi bodies appear

as jet-black grains, the normal Golgi bodies as fine curved black rodlets, and the yolk spheres as yellowish-brown spheres.

On the application of Baker's acid-haematein technique it was possible to detect in the cytoplasm numerous small blue-black granules corresponding to the mitochondria, a few slim dark-blue rodlets probably corresponding to the unchanged Golgi bodies, many large pale bluish-brown spheres corresponding to the proteid yolk spheres, and lastly yellowish spheres that correspond to the fat-loaded Golgi bodies.

When the acid-haematin test is applied after pyridine extraction, only the nucleoli inside the nucleus and the proteid yolk spheres in the ooplasm gave positive reaction, the nucleolus being stained a much deeper blue than the yolk spheres.

The acid-haematin/pyridine extraction combination, therefore, shows that lipin is not present in any appreciable concentration in the desert snail's oocytes except in the mitochondria and the unchanged Golgi bodies.

The Centrifuged Oocyte

In the fully grown oocyte, the different ooplasmic elements are more or less haphazardly mixed. On centrifuging, however, it was found that these elements separated in successive strata. Naturally this gives a better chance for the determination of the physical and histochemical characteristics of the different elements in each stratum.

The ovotestis was immersed in an isotonic Ringer's solution or snail's blood, and then centrifuged at a speed of 3,500 revolutions per minute for half an hour. Immediately after centrifuging, the serum or Ringer was poured off, and the fixatives instantaneously applied. The centrifuged material was treated with mitochondrial and Golgi techniques and with Baker's formol-calcium/acid-haematein method.

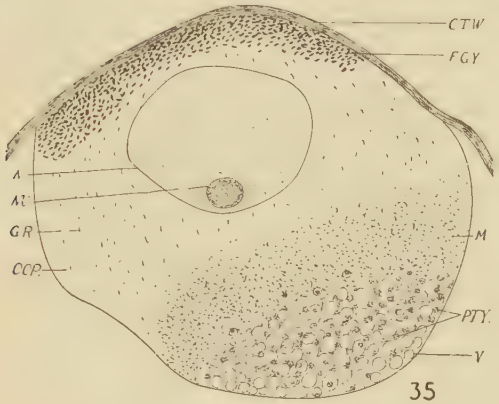
The cytoplasmic bodies of the centrifuged oocyte (Text-fig. 35) appear stratified in four layers. The uppermost layer occupies 10 per cent. of the volume of the oocyte, and contains oval, fairly large bodies. These bodies reduce osmic acid easily, appearing jet-black after post-osmication and even in unstained chrome-osmium sections. In formalin-fixed material, subsequently stained in Sudan III or Scharlach R, these bodies stained brilliant red. There is little doubt, therefore, that this layer represents the fat-loaded Golgi elements (Text-fig. 35, F.G.Y.).

Just underneath the above-mentioned layer there appears a layer of clear cytoplasm occupying about 50 per cent. of the volume of the oocyte and containing several unevenly scattered Golgi rods (Text-fig. 35, OOP). In this layer the nucleus lies with the nucleolus shifted towards the centrifugal pole. Sometimes the nucleolus shattered the nuclear membrane and became thrown off for a short distance into the underlying cytoplasm.

The third layer appears as a band of granules extending underneath the clear area. These granules appear yellowish in unstained chrome-osmium and Mann-Kopsch preparations, but stain strongly in Altmann's acid fuchsin,

crystal violet, iron haematoxylin, and Baker's acid haematein. This layer is, therefore, undoubtedly mitochondrial (Text-fig. 35, M).

The heaviest layer at the centrifugal pole of the oocyte shows a collection of vacuoles and numerous coarse and fine spherules. The contents of the vacuoles give negative tests for fats, proteins, and glycogen. It seems, therefore, that their contents are essentially watery. The spherules appear yellowish-brown in unstained Nassonov and Mann-Kopsch preparations, and stain weakly or not at all in Altmann's acid fuchsin and haematoxylin and



TEXT-FIG. 35. The centrifuged oocyte.
From Nassonov/Altmann's fuchsin/aurantia preparations. ×340. Lettering as before.

Baker's haematein. There is little doubt, therefore, that these represent the true yolk spheres (Text-fig. 35, PT.Y).

In the mitochondrial and yolky strata one may occasionally find a few Golgi rods. These seem to have been entangled with the granular elements (mitochondria and yolk spheres) in their centrifugal drift.

Brambell (1924), after centrifuging the oocytes of *Helix aspersa*, found a single layer of swollen mitochondria at the centrifugal pole. These he took to represent what he called 'mitochondrial yolk'.

The centrifuged oocyte of the desert snail shows towards the centrifugal pole two layers and not one: an upper layer of mitochondrial granules of various sizes and a lower one of true yolk spheres. The latter, as previously shown, have nothing to do with the mitochondria, being formed under the influence of the Golgi bodies.

DISCUSSION

During the last 25 years, cytological literature has been full of valuable publications seeking to disclose the mechanism of yolk formation in growing oocytes. At present there are two competing schools of thought.

According to one school (Nath and co-workers, 1924 et seq.), fat is formed in relation to the Golgi bodies, whereas yolk is derived from nucleolar extrusions

or arises *per se* in the ground cytoplasm. This method of deutoplasmogenesis was described in Crustaceans (*Palaemon*—Bhatia and Nath, 1931, and *Paratelphusa*—Nath, 1934); Chilopods (*Lithobius*—Nath, 1924, and *Otostigmus*—Nath and Husain, 1928); Spiders (*Crossopriza*—Nath, 1928, and *Plexippus*—Nath, 1934); Insects (*Luciola*—Nath and Mehta, 1929, *Periplaneta*—Nath and Piare Mohan, 1929, and *Culex*—Nath, 1929); Fishes (*Rita* and *Ophiocephalus*—Nath and Nagia, 1931, and also Nath, 1934); Amphibia (*Rana*—Nath, 1931 and 1934); Reptiles (*Emyda*—Nath and Azez Ahmed; quoted in Nath, 1934) and Birds (*Gallus*—Nath, 1934).

The Golgi bodies, according to these authors, are in the shape of vesicles, with osmiophil rims and osmiophobe cores. At some stages in oogenesis fat is deposited within the Golgi vesicles, thus causing them to swell into the fatty yolk spheres (see especially Nath, 1930).

Applying Scharlach R and Sudan III on fresh and formalin-fixed oocytes, Nath (1934) could, in several instances, stain his 'Golgi spherules' red in the older but not in the younger oocytes. This he considered as further evidence in favour of the Golgi origin of fatty yolk.

Of the upholders of the principle of origination of fat in connexion with Golgi bodies, apart from Nath's school, may be cited King (1926), working on *Oniscus*; Gresson (1929, 1931, and 1933), working on three species of Tenthredinidae and also *Periplaneta orientalis* and *Stenophylax stallatus*, and also Bell (1929), who showed that even in the male germ cells fat may be derived from the Golgi bodies.

On the other hand, a group of competent observers have maintained that the Golgi bodies are somehow concerned in the production of true (proteid) yolk.

Wheeler (1924) working on *Pleuronectus*, Weiner (1925) on *Lithobius* and *Tegeneria*, and Steopoe (1926) on *Nepta* have all found that yolk is formed in the periphery of the oocyte among and in intimate relation to the Golgi bodies. Also Gardiner (1927), working on *Limulus*, found that yolk appears in regions of the ooplasm where Golgi bodies and mitochondria are maximally aggregated. He suggested that yolk arose through the interaction of mitochondria, Golgi bodies, and nucleolar extrusions. To Harvey, L. A. (1929 and 1931), the credit of establishing the role of the Golgi bodies in the production of true yolk must be attributed. In his works on *Carcinus* and also *Antedon* and *Asterias*, he maintains that the raw material of this yolk is largely derived from the exterior of the oocyte, but it is also partially provided by the nucleolar extrusions. From this raw material yolk is synthesized, presumably through the activity of mitochondria, and then becomes condensed under the influence of the scale-like Golgi bodies into droplet form, the process being described as 'physical condensation rather than chemical synthesis'. As to fat, Harvey believes that it arises *per se* in the ground cytoplasm.

In the present work on the desert snail, it was found that true yolk (largely proteid) originates under the influence of the Golgi elements. Frequently the primordium of the yolk sphere appears in the interior of a Golgi circle,

formed by the grouping of some Golgi rods end to end. This positional relationship between the early yolk spheres and the Golgi rods indicates, as Harvey (1929) notes for similar conditions in *Carcinus*, 'that the Golgi elements are mostly concerned with the final stages of condensation of yolk in the cytoplasm, whatever may be their relations in yolk synthesis' (loc. cit., p. 168).

The claims of Brambell (1924) that some of the mitochondrial elements of the oocytes of *Helix* and *Patella* swell into 'mitochondrial yolk' have nothing to uphold them from the present study. Late in growth, mitochondrial granules grew almost imperceptibly in size, but still remained very much smaller than the smallest yolk spherules. Further, all through vitellogenesis the mitochondrial granules preserved the histochemical characteristics of mitochondria and reacted to stains and fixatives differently from yolk. Harvey (1929) favoured the possibility that the role of mitochondria in vitellogenesis is not the final condensation of the yolk spherules, but rather the synthesis of the definitive yolk molecules from the raw material prevailing in the ooplasm. This is probably the case in the desert snail as well, since no positional relationship occurred between the mitochondrial elements and the developing yolk spherules.

Nucleolar extrusions were described in Mollusc oocytes only by Ludford (1921*b*). However, Gatenby (1917), and even earlier Ancel (1903) detected in the cytoplasm of the oocytes of Helicids certain masses which disappeared late in growth. The present work on the desert snail revealed that similar masses are in all probability nucleolar extrusions. These subsequently disappear, their material probably providing at least some of the raw material used in yolk synthesis.

Fat, in the oocytes of the desert snail, deposits on the Golgi rods, after the latter have left the yolk spheres and clumped in groups especially towards the periphery of the ooplasm. Brambell's (1924) claim that fat (his Golgi yolk) in the oocytes of *Helix aspersa* arises through direct metamorphosis of the Golgi rods is not substantiated by the present work. Careful histochemical tests (pp. 173–5) showed that the definitive Golgi rod always preserved its identity and only became loaded with a variable amount of free fat.

The course of vitellogenesis in the desert snail serves marvellously in the settlement of the long-lived controversy between L. A. Harvey and Nath and co-workers as to the role of the Golgi bodies during vitellogenesis. In agreement with Harvey's view, true yolk in the desert snail's oocyte arises under the influence of the Golgi rods. After these rods are released from this function, they become loaded with fat, a fact in harmony with the essence of the view put forward and defended by Nath's school.

Acknowledgements

The present problem has been suggested by and undertaken under the direction of Professor K. Mansour, Head of the Department of Zoology, Fouad I University, Cairo. His advice on technique and his many valuable suggestions are gratefully acknowledged.

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Summary

1. The histological structure of the ovotestis is briefly described.

2. The germinal epithelium gives rise to both categories of germ cells (male and female) as well as to the nurse cells.

3. During the differentiation of the germinal epithelial cell towards the germ line, it passes through a certain stage, 'the progerminative indifferent stage', before it is polarized either towards the male or female line.

4. A detailed description of the mode of differentiation of the progerminative indifferent cell into the earliest female element is given.

5. Mitotic multiplication of the early female elements never occurs. Nevertheless the diploid chromosome complement is represented by prochromosomes.

6. During the oocytic meiotic prophases, parasynaptic conjugation of the chromosomes is quickly followed by relational coiling of the synaptic mates and the formation of the strepsitene double spirals.

7. The strepsitene double spirals give rise to the diplotene bivalents of the haploid count 28.

8. The steps of chromatin diffusion that lead to the construction of the typical oocytic germinal vesicle are described.

9. Changes in size, number, and stainability of nucleoli during oocytic growth are recorded. Extrusion of basiphil material from the nucleolus into the ooplasm is highly probable.

10. The mitochondrial granules at the beginning of the period of active oocytic growth form a huge zone to one side of the nucleus. Later they increase in number and widely scatter in the ooplasm. It is held (in favour of Harvey's view, 1929 and 1931) that mitochondria are probably concerned in the chemical synthesis of yolk from raw material provided in the ooplasm.

11. The Golgi bodies in oocytes are in the form of rods and not vesicular. In the early oocyte they form a group to one side of the nucleus. Eventually they disperse in the ooplasm either singly or in small typical complexes. Each of the latter is constituted of a few rods arranged end to end as if on the perimeter of an irregular circle.

12. The earliest yolk spheres (largely proteid) appear in the interior of the Golgi complexes or on the concave sides of the separate Golgi rods.

13. The Golgi rods become severed from the yolk spheres and migrate mainly towards the periphery of the oocyte where they collect in clumps.

14. The elements of the Golgi clumps become loaded with an unsaturated free fat. The original Golgi rod always preserves its identity and never transforms itself into fat.

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The Distribution of Alkaline Phosphatase in the Skull of the Developing Trout

BY

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(With two Plates)

Introduction

THIS work is part of a comparative study of the histological and cytological distribution of alkaline phosphatase in developing teleost and elasmobranch fishes, made with a view to throwing some light on the mechanisms involved in calcification and ossification. Whereas the adult mammalian skeleton is almost completely ossified, in the lower vertebrates cartilage persists side by side with bone. In elasmobranchs bone does not occur but calcium deposits are formed in the cartilage. Here calcification can be studied independently from ossification.

No histochemical study of phosphatase in lower vertebrates has so far been published. There is, however, evidence that phosphatase exists in teleosts as well as elasmobranchs, and is closely similar to the alkaline phosphatase found in ossifying parts of mammals (Bodansky, Bakwin, and Bakwin, 1931; Roche and Bullinger, 1939). The latter authors found a correlation between the degree of ossification and the phosphatase content in different species of fish and at different stages of development in the same species. A study of phosphatase in relation to the growth of scales in elasmobranchs and teleosts leads Roche, Collet, and Mourgue (1940) to conclude that here, as in the higher vertebrates, phosphatase is concerned with rapid osteogenesis.

These results suggest that the same biological mechanism is operative in the calcification of the skeleton in fish as in mammals. In view of this a systematic study of the distribution of phosphatase and calcium salts in developing teleost and elasmobranch embryos seemed of interest. This incidentally provides material for the study of phosphatase in the embryo generally. Studies so far undertaken in this direction were confined to young fowl embryos (Moog, 1944) and the heads of rat embryos (Horowitz, 1942).

This paper deals only with ossification in a typical teleost. The results obtained with elasmobranch embryos will be published separately. The trout (*Salmo* spp.) was chosen because it can conveniently be reared in the laboratory. Moreover the development of *Salmo* has been intensively studied from

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the morphological point of view (Schleip, 1903; Gaupp, 1906; Böker, 1913; Saunderson, 1935; de Beer, 1927 and 1937). Emphasis is here placed on the phosphatase content and degree of calcification rather than on the anatomical aspects of bone formation.

In the trout as in all teleosts, bones arise in a variety of ways. They may on the whole be classified into membrane bones, cartilage bones, and mixed ossifications.

I am here mainly concerned with the early stages of bone formation, i.e. the formation of the pre-osseous matrix—either within the mesenchyme as in membrane bone formation, or below the perichondrium as in cartilage bone formation—and the subsequent calcification of the matrix with its accompanying variations in phosphatase content and distribution. Examples of the different types of ossification will be described in detail. There was complete agreement of the phosphatase distribution and general appearance among fish at the same stage of development (not necessarily of the same age, but usually of the same length). The structures described were therefore chosen from whichever specimens showed the particular features best. The nomenclature used is that of de Beer (1937).

The following cartilages are suitable for the study of phosphatase in relation to their development and ossification:

Neurocranium. The anterior wall of the auditory capsule and the lateral commissure give rise to the pro-otic bone which later involves the basal plate and the anterior end of the parachordals. The basi-occipital appears in the form of perichondral lamellae of the hind portion of the basal plate on each side of the notochord.

Splanchnocranium. Meckel's cartilage and the autodentary; the pterygo-quadrate giving rise to the autopalatine, the metapterygoid, and the quadrate; the hyosymplectic cartilage giving rise to the hyomadibula and the symplectic; other visceral cartilages.

The following structures illustrate *membrane bone* development: (i) the parasphenoid, a typical flat membrane bone which develops in the mesenchyme between the trabecula and the mucous membrane of the mouth. (ii) The dermodentary, i.e. the membrane bone portion of the dentary which is a mixed ossification. Since it develops in close relationship to Meckel's cartilage the structures composing the lower jaw will be described together after Stage 1. (iii) The maxilla, a membrane bone of the upper jaw entering into relationship with teeth. The premaxilla develops on similar lines. (iv) The pre-opercular which lodges part of the mandibular lateral line canal and lies postero-lateral to the symplectic cartilage.

Details of the above structures are described in my thesis (Lorch, 1948). Here only examples of the different types and stages of ossification are given.

The terms 'positive', 'strongly staining', 'black', or 'grey' refer to the presence of phosphatase. No attempt has been made to give a strictly quantitative estimate of the phosphatase content of the tissues. The degree of staining after various incubation times is the sole criterion for stating that a tissue is

'strongly positive' or 'contains little phosphatase'. A 'negative' Gomori reaction does not necessarily imply complete absence of the enzyme since there is a considerable loss during the preparation of the tissues.

Material

Specimens of brown and rainbow trout were reared from 'eyed' ova in the laboratory. It was decided to use length rather than time from hatching as an indicator of development. About 40 specimens ranging from 10 to 40 mm. were examined.

It was found convenient to divide the developing trout into groups of approximately the same length as follows:

Stage (for reference)	*Length (mm.)*	*No. of specimens examined*
1	10–12	6
2	15·5–17	5
3	20	6
4	21–23	11
5	29–38	10

Methods

Samples of trout were removed at intervals after hatching. Any yolk present was dissected off and the length of the specimen measured to the nearest millimetre. Younger alevins were anaesthetized in 10 per cent. alcohol before fixation to prevent curling and to facilitate measurements. Specimens intended for visualization of phosphatase were fixed in 80 per cent. ethyl alcohol. Some specimens of each stage were fixed in Bouin's fluid for general histology and some in 5 per cent. neutral formalin for the preparation of whole mounts of the skeleton stained with alizarin red S by the method of Hollister (1934). The latter helped in identifying the calcified structures and gave an indication of the areas in which calcification first occurred.

Most of the specimens intended for the phosphatase technique were cut undecalcified. Some of the older specimens were decalcified using citrate buffer for 2 to 5 hours according to my method (Lorch, 1947). There was a slight reduction in the phosphatase content. Groups of serial sections (8–10μ) were mounted on alternate slides, one being incubated and the other used as control. The slides were taken through celloidin to distilled water. Phosphatase was visualized by the method of Gomori (1939) and Takamatsu (1939). Minor changes in the composition of the substrate were made as follows:

2 per cent. calcium nitrate 10 ml., 2 per cent. magnesium chloride 10 ml., 4 per cent. sodium β-glycerophosphate 10 ml., 1 per cent. sodium barbitone 70 ml.

Slides were incubated 1–18 hours at 28° C. and pH 9·4. Subsequent treatment was as described by Danielli (1946). Some sections were counterstained with dilute eosin.

Where the amount of preformed phosphate was considerable (specimens over 20 mm.) it became desirable to show the calcium salts and phosphatase in different colours, hence the gallamine blue technique was applied. The reasons for choosing this method as well as its limitations are discussed in my paper on mammalian bones (Lorch, 1947).

The following histological stains were used: Heidenhain's 'Azan', van Gieson's connective tissue stain, Ehrlich's haematoxylin and eosin, and von Kossa's silver nitrate method for bone salts.

Results

Stage 1 (10–12 mm.)

A. *General Distribution of Phosphatase*

In all tissues which contain phosphatase the reaction is most marked in the nuclear membranes and nucleoli. The cytoplasm on the whole is negative, but it is not always easily recognizable since the degree of shrinkage and distortion due to alcohol fixation is considerable in the younger embryos. The mesenchyme stains irregularly—nearly all areas show some degree of activity—and the most marked concentration of positive nuclei occurs at the angles of the mouth and in the vicinity of the jaw cartilages. The central nervous system displays positive nuclei and fibres; the intensity of the reaction is variable. The fibrous membranes of the brain are strongly positive. The cells of the retina display slightly positive nuclei at some levels. Nuclei of striated muscle, and of the endothelial cells lining blood-vessels, are positive. The epithelium is negative.

B. *Skeletal Tissues*

All the cartilages of the chondrocranium display nuclear phosphatase, more or less marked, and in some places the matrix is also slightly positive, more so in the 10-mm. than in the 12-mm. specimens.

Neurocranium. Anterior to the region of the articulation of the lower jaw only the chondrocyte nuclei in the trabeculae are positive, but posteriorly, i.e. near the parachordals, the matrix too displays slight phosphatase activity. The cartilage cells are large and the area occupied by matrix relatively very small. The nuclei have a granular appearance. The cytoplasm is very faintly positive, and can just be observed in slides incubated 15 hours. The perichondral fibroblasts are strongly positive and fine black fibrils are seen in the surrounding mesenchyme which stains most strongly dorsally to the trabecula (Pl. 1, fig. 1). The parachordals contain strongly positive nuclei. The matrix stains positive in well-defined zones where the cartilage is cut near its surface. In the auditory capsules only the nuclei are positive. The perichondrium is still strongly positive on the dorsal (brain) side. More distally the para-

chordals lose their extracellular phosphatase except in the zone adjacent to the notochord: the nuclei remain positive throughout.

The notochord sheath consists of 3 layers: an inner layer of cubical cells with strongly staining nuclei, a middle layer, seemingly structureless and free from phosphatase, and an outer layer of elongated fibroblasts closely packed and strongly positive. The substance of the notochord itself is free from phosphatase.

Splanchnocranium. Meckel's cartilage is completely negative in its anterior tip where the pair of cartilages join. The middle and posterior regions contain nuclear phosphatase, the enzyme being most concentrated in the nucleoli and nuclear membranes. There is no extracellular phosphatase in Meckel's cartilage. The distribution of phosphatase in the mesenchymal nuclei of the lower jaw is interesting: they are strongly positive lateral to, moderately so ventral to, and negative dorsal to Meckel's cartilage. This is shown in Text-fig. 1, and will be further discussed under membrane bone development.

Pl. 1, fig. 2, shows the quadrate process and the hyosymplectic cartilage of a 12-mm. specimen. The mesenchymal nuclei are strongly positive. The hyosymplectic also shows nuclear phosphatase and some extracellular phosphatase at the periphery. The basihyal displays positive nuclei only after long incubation times, while the other branchial cartilages show strongly staining chondrocytes and perichondral fibroblasts after 6 hours' incubation. There is phosphatase in the striated muscle nuclei.

The first stages in the formation of *membrane bone* are illustrated by the dermodentary. This is seen as a minute fragment of uncalcified osteoid at some levels only. It forms a thin lamella central and lateral to Meckel's cartilage, i.e. in the region where the mesenchymal nuclei are most strongly positive. The osteoid contains phosphatase (Text-fig. 1).

The pre-opercular resembles the dermodentary. The maxilla is present as a very small incompletely calcified rod angular in cross-section. There is as yet no sign of the parasphenoid.

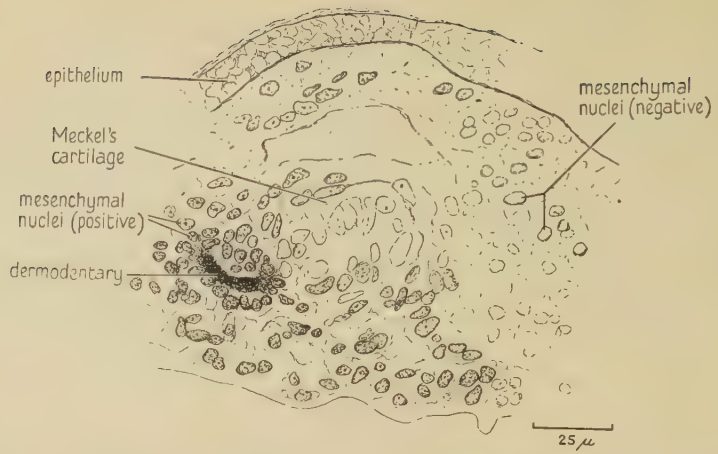
Summary of Stage 1

Nuclear phosphatase is widespread in skeletal as well as non-skeletal tissues. There are occasional traces of enzyme in the cartilage matrix. Osteoid is present in some phosphatase-rich areas of mesenchyme. There is no calcification.

Stage 2 (15·5–17 mm.)

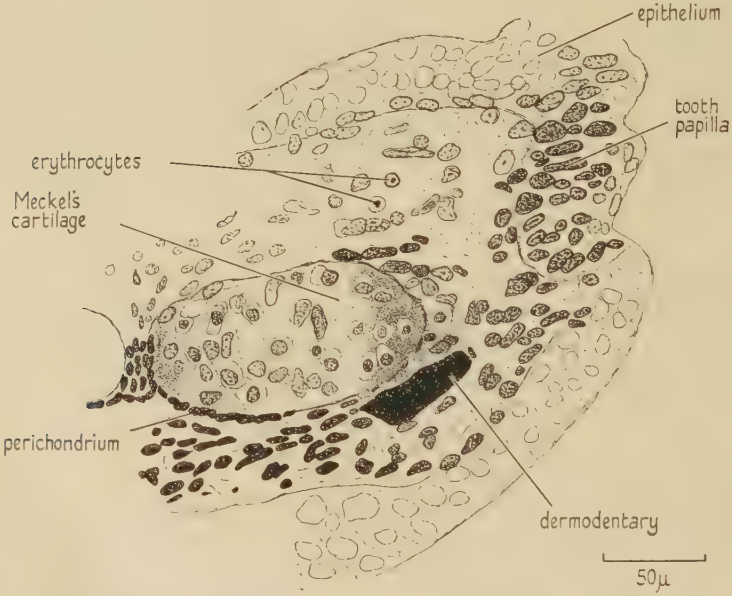
A. *General Distribution of Phosphatase*

This does not differ greatly from the distribution at the previous stage. But a new zone of strongly positive mesenchyme is seen just below the epithelium of the lower jaw anterior to the basihyal, and between the anterior end of the basihyal and the mouth epithelium. It is interesting to note that this is the site of the future dermentoglossum bone and tooth buds. Another intensely active zone of mesenchyme is that above and below the trabecula communis. In the latter zone the parasphenoid has its origin.



TEXT-FIG. 1. From the lower jaw of a 12-mm. trout. Incubation time 6 hours. The highest concentration of phosphatase in the mesenchymal nuclei is in the vicinity of the dentary. No tooth papillae are formed yet. The epithelium is negative. The dentary is not calcified. Cf. Text-fig. 2.

All text-figs. except diagrams are camera-lucida drawings of undecalcified sections treated by the Gomori (1939) method, unless otherwise stated. Areas of phosphatase activity are shown black.



TEXT-FIG. 2. From the lower jaw of a 16-mm. trout. Incubation time 15 hours. The pair of Meckel's cartilages are just separated. The dermodentary is very close to the cartilage but not in apposition. Note the positive reaction of the mesenchymal nuclei and of the epithelium in the region of the tooth papilla. The cartilage matrix is slightly positive. The dentary is calcified.

B. *Skeletal Tissues*

In embryos of about 17 mm. the chondrocranium has almost reached its full development. The trabeculae, and the anterior part of the parachordals, display little or no phosphatase activity except in the dorsal perichondrium, and this applies to all subsequent stages. These parts do not ossify.

There is a strongly positive reaction of the cartilage matrix in apposition to the notochord, in the region of the future basioccipital. The auditory capsules display little or no phosphatase apart from the perichondrium.

Splanchnocranium. The extreme tip of Meckel's cartilage is still not calcified. It shows phosphatase in the nuclei and also some in the matrix, especially at the periphery, where a thin layer of osteoid, the first sign of the autodentary, can be seen. The dermodentary is first visible at a level where the two cartilages just separate (Text-fig. 2). This stage shows the earliest calcification in the dentary. It is interesting to observe that the ground substance of the bone here contains phosphatase (visualized in decalcified sections), whereas in the older specimens this is rarely the case. Only the central portion of the bone is calcified as shown by treatment with silver nitrate. A rim of osteoid remains at the periphery of all growing bones. In Text-fig. 4 Meckel's cartilage and the dentary are cut longitudinally. Calcification decreases in intensity towards the distal end of the bone, which consists of a shread of uncalcified osteoid. The mesenchymal cells and fibres surrounding the dentary are strongly positive in this region. Meckel's cartilage has positive nuclei throughout but extracellular phosphatase only at the anterior end, i.e. where the dentary is in apposition to it. There are no new developments in the other branchial cartilages.

Membrane Bone. The dermodentary has already been described and its appearance is typical for all membrane bones at an early stage in their formation. The appearance of the maxilla is as at Stage 1. The parasphenoid is now seen ventral to the trabecula communis. With regard to the presence of calcium salts and phosphatase it resembles the early dermodentary.

Summary of Stage 2

The general distribution of phosphatase does not differ greatly from that at Stage 1. The perichondrium of some cartilages is strongly positive. Extracellular phosphatase occurs in the cartilage matrix where perichondral osteoid is present or about to be formed, e.g. at the anterior end of Meckel's cartilage. The first stages of calcification are observed in membrane bones.

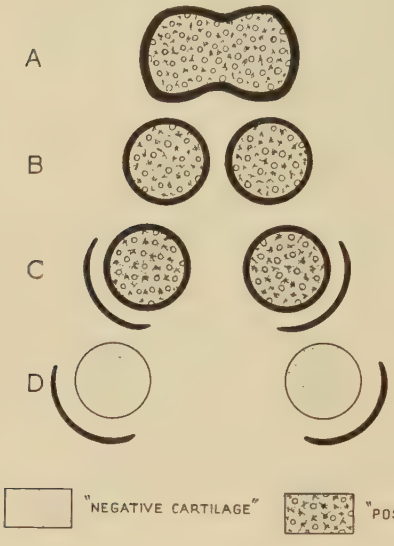
Stage 3 (20 mm.)

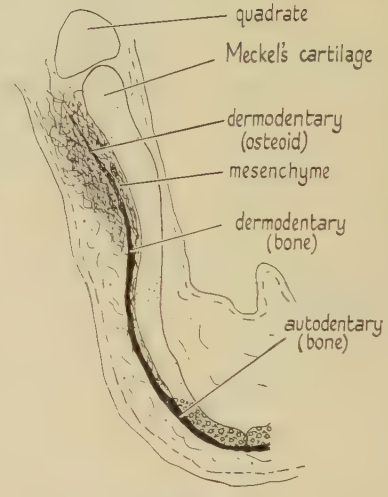
A. *General Distribution of Phosphatase*

The appearance of the central nervous system is unchanged. The retina displays a well-localized zone of high phosphatase activity in the nuclei of the light-receptive cells. The reaction is somewhat weaker at the periphery of the retina than in the central region. The lens epithelium is positive. The

sensory epithelium of the nasal grooves has positive nuclei, and the fine cilia protruding from the olfactory cells stain heavily. The epithelium of the mouth and skin is negative.

Phosphatase in the mesenchyme is localized in the zones described for the previous stage. Tooth buds have now appeared at the angles of the mouth and in the strongly positive zone above the basihyal. The epithelium is





TEXT-FIG. 3 TEXT-FIG. 4

TEXT-FIG. 3. Diagrammatic representation of transverse sections through Meckel's cartilage and dentary in different regions. A. Proximal tip. Cartilages fused and surrounded by the autodentary cartilages of 'positive type'. B. Cartilages just separated. 'Positive type.' C. Slightly distal to B. Proximal part of dermodentary overlapping with posterior part of autodentary. Cartilage still 'positive'. D. Distal to C only the dermodentary is seen. Cartilage of 'negative type'.

TEXT-FIG. 4. Diagrammatic representation of a longitudinal section through the lower jaw of a 16-mm. trout showing the distribution of phosphatase in Meckel's cartilage and the dentary. The latter is calcified only in its anterior portion. The posterior portion consists of osteoid and is partly obscured by strongly positive mesenchyme.

The key to the shading applies to all subsequent figures in which a diagrammatic style is used.

thrown into folds and papillae of the strongly staining mesenchyme project into it.

As in previous stages the mesenchyme surrounding the growing membrane bones is strongly positive.

B. *Skeletal Tissues*

The general distribution of phosphatase within the chondrocranium tends towards more localization of the enzyme in certain areas and reduction in others. Whereas in Stage 1 all cartilages have positive nuclei this is no longer the case; some are completely negative, others have very high concentrations

of phosphatase in both nuclei and matrix. As will be seen below this accumulation of enzyme can always be correlated with the development of a bony shell round the cartilage. Pl. 1, fig. 3, shows that the hyomandibula is positive in patches and the ceratohyal stains intensely. Portions of the chondrocranium which serve as attachment for muscles tend to give a positive reaction. There is a strongly staining region in the walls of the foramen of the facial nerve where the pro-otic bone can be seen in slightly older specimens (Stage 4).

Splanchnocranium. The extreme anterior end of Meckel's cartilage is now completely surrounded by a shell of bone, the mentomeckelian ossification, which merges into the autodentary posteriorly. The chondrocytes in the anterior portion show strongly positive nuclei and there is some phosphatase in the matrix, especially at the point where the pair of cartilages is just separating. The intensity of the phosphatase reaction decreases from the strong nuclear and extracellular reaction in the anterior tip to a weak nuclear reaction at a level where the cartilages are widely separated and the dentary is merging from its cartilage bone portion into the membranous portion. Distal to this the cartilage is completely negative apart from a few isolated nuclei chiefly at the periphery.

The appearance of the cartilage varies in the regions of different phosphatase activity: in the anterior portion the chondrocytes are large and spherical, their nuclei appear either completely black or granular, the cytoplasm gives a positive reaction, and the cells are surrounded by a rim of strongly staining ground substance, the rest of the matrix being moderately positive. In the eye region where the cartilage is negative the chondrocytes are arranged in longitudinal rows and are bilaterally compressed, in contrast to the spherical cells of the anterior zone. In the region of the optic chiasma the thick outer lamella of the dermodentary is fully calcified, while the inner thin lamella consists of uncalcified or feebly calcified osteoid. Both parts are embedded in strongly positive mesenchyme.

The Pterygoquadrate. The chondrocyte nuclei of the pterygoid process are moderately positive on the dorsal and external surface, i.e. where the metapterygoid bone is due to develop. In some of the specimens the endopterygoid (membrane bone) is seen as a thin layer of osteoid central to the inner perichondrum of the pterygoid process. It is not yet calcified and gives a positive phosphatase reaction.

In the region of its articulation with Meckel's cartilage and with the hyosymplectic cartilage the appearance of the quadrate process changes to that described for the anterior portion of Meckel's cartilage, and this is typical for all ossifying zones. The extracellular phosphatase activity is most marked near the lateral edge of the cartilage where a broad zone of feebly calcified osteoid, the first sign of the quadrate bone, can now be seen (Pl. 1, fig. 4). Phosphatase is never found in the matrix of cartilages which have no perichondral layer of osteoid or bone. Thus the characteristic features of ossifying cartilage in the trout are: (*a*) Greatly enlarged spherical cells with round nuclei, which later appear to degenerate and stain only faintly with haematoxylin.

These cells have very marked phosphatase activity. (*b*) The presence of phosphatase in the cartilage matrix, especially in the newly secreted ground substance round the chondrocytes which therefore appear to have black 'haloes'. The matrix is strongly basiphilic and in staining reaction resembles mammalian hypertrophic cartilage. However, no calcification of the matrix itself has been observed.

Since this type of cartilage is constantly met with, and always in conjunction with perichondral bone formation, it will be briefly referred to as 'positive cartilage', the above characteristics (including bone or osteoid) being implied in that expression. The term will not be used to describe cartilage containing phosphatase in the cells and perichondrium only. In diagrams of sections 'positive cartilage' is represented by areas with black rings, whereas 'negative cartilage' is stippled. Bone or osteoid is shaded. Thus the appearance of Meckel's cartilage described on p. 191 may be represented by a series of diagrams shown in Text-fig. 3.

In specimens of 15 mm. or over all cartilages fall into one of the following categories: (*a*) cartilages displaying no phosphatase activity whatever, (*b*) cartilages displaying some phosphatase in nuclei and perichondrium, and (*c*) cartilages with considerable phosphatase activity in cells, perichondrium, and matrix ('positive type'). Only the latter are ossifying.

The Hyoid Arch. The anterior zone of the symplectic process has the same appearance as the quadrate in this region, i.e. the perichondrium and chondrocyte nuclei are positive. Slightly distal to this there is an abrupt transition to the 'positive type' of cartilage. This is the first sign of the symplectic bone which ossifies in the distal portion of the hyosymplectic cartilage. Pl. 1, fig. 5, shows the posterior end of the quadrate and immediately below it the hyosymplectic cartilage with its layer of bone. The contrast between the adjacent cartilages is very marked.

The hyomandibular portion of the cartilage is not yet calcified but shows a thin perichondral lamella of pre-osseous tissue. The perichondrium is strongly positive especially where the cartilage is pierced by the hyomandibular branch of the facial nerve. The chondrocyte nuclei are positive throughout. A few enlarged cells of the type found in ossifying cartilages are seen and are surrounded by positively staining 'haloes' of ground substance. The mesenchyme enclosing the pre-opercular bone is intensely positive.

Other Branchial Cartilages. The ceratohyal is partly surrounded by a thin shell of bone (the epihyal) and presents the usual picture of an ossifying cartilage. No other cartilages have extracellular phosphatase, but some display positive nuclei and perichondrium.

Membrane Bone. The dermodentary has been described in conjunction with Meckel's cartilage. The maxilla is now heavily calcified especially in its anterior portion and is best studied in decalcified sections where its relationship to the surrounding mesenchyme is more clearly seen. It may be described in some detail as typical of membrane bone at this stage of development. In sections incubated for 14–16 hours the very heavy deposit of calcium salts

obscures the individual cells since the mesenchyme at the angles of the mouth is strongly positive. But in sections incubated for 4–6 hours only, the cells close to the maxilla (or other membrane bone studied) are positive, hence these cells have the highest concentration of phosphatase. The bone itself is negative after this incubation time but may appear grey after 18 hours. Three types of cells can be distinguished in the vicinity of the bone: long fibroblasts with narrow nuclei, large oval cells with prominent nuclei, and polymorphous cells of an intermediate size. The latter make up the bulk of the mesenchymal cells. The large cells are found close to the bone. They may be osteoblasts. The phosphatase content of these cell types is very variable. The cytoplasm only displays phosphatase after very long incubation times. The fibroblast nuclei stain deep black, most other nuclei appear granular. Nucleoli and nuclear membranes are prominent in all cell types. Text-fig. 5 shows a typical field of membrane bone and surrounding cells. In the bone itself two regions of different refractive index can sometimes be distinguished: a central and peripheral zone. In stained preparations, too, the heterogeneous nature of the ground substance becomes evident, the older ground substance having a different appearance from that newly secreted.

Summary of Stage 3

Ossifying cartilages can be distinguished from non-ossifying ones by the presence of high concentrations of phosphatase in the matrix as well as in the enlarged chondrocytes. In non-ossifying cartilages phosphatase, if present, is confined to the nuclei. Membrane bones display phosphatase only in the newly formed portions.

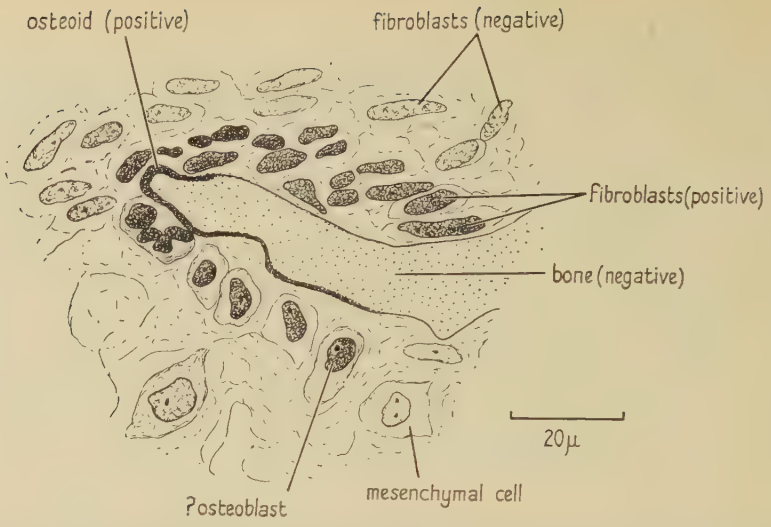
Stage 4 (21–3 mm.)

A. *General Distribution of Phosphatase*

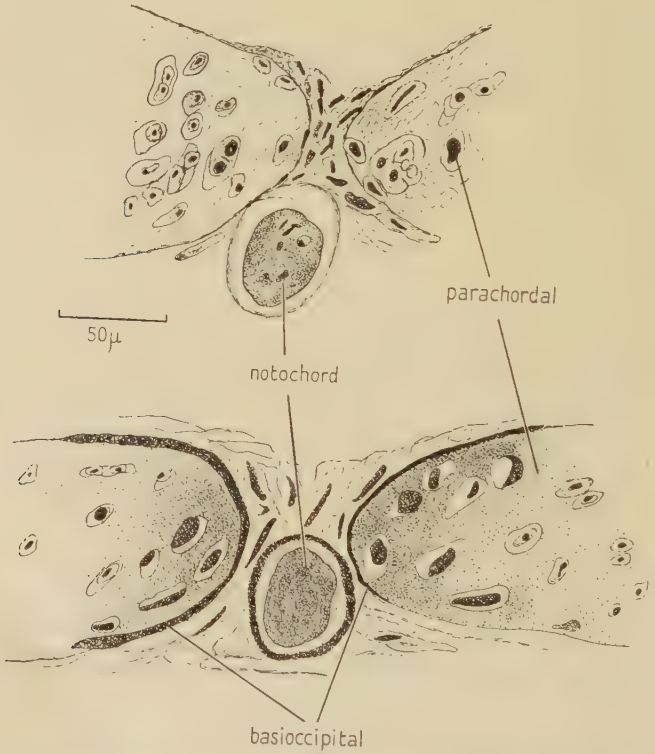
The decline of nuclear phosphatase in tissues such as muscle, peripheral nerves, mesenchyme, and non-calcifying cartilage, already noted at the previous stage, continues. The skin and mouth epithelium, except at the tip of the lower jaw, remain negative. The anterior region of the brain, the retina, lens epithelium, and the nasal mucosae are strongly positive. The reaction becomes weaker in the mid-brain, the rest of the central nervous system being only feeble positive.

B. *Skeletal Tissues*

At the exit of the facial nerve, where the pro-otic is now seen in the form of two perichondral lamellae, the anterior wall of the auditory capsule is positive in cells and matrix. The bony lamellae, like all perichondral ossifications, are homogeneous and sharply separated from the cartilage as well as from the connective tissue. (Appearance similar to parachordals in Text-fig. 6.) A few small spindly shaped cells form the periosteum. Ossification of the lateral commissure and the walls of the foramen for the trigeminal nerve has also begun.

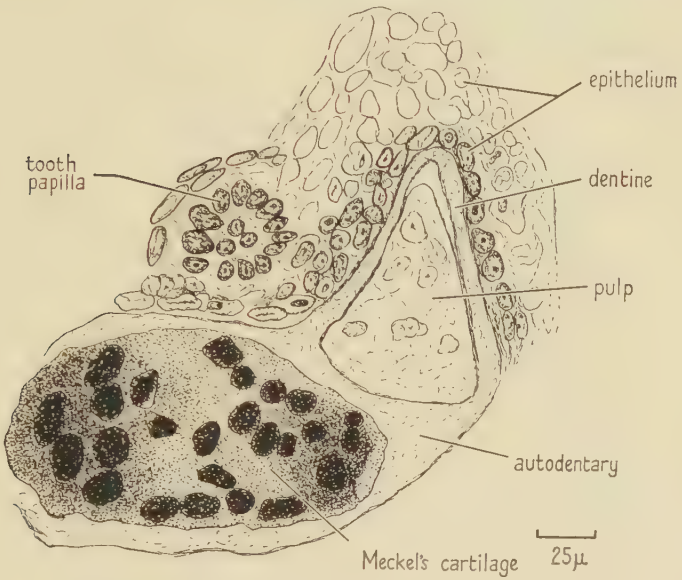


TEXT-FIG. 5. Membrane bone and associated cell types of a 20-mm. trout. Decalcified section. Incubation time 15 hours. Note that only the growing-tip contains phosphatase.



TEXT-FIG. 6. Transverse sections through the notochord, parachordals, and basi-occipital of a 23-mm. trout. Incubation time 15 hours. Section (*b*) is slightly distal to (*a*). The perichondral lamellae of the basi-occipital are seen in (*b*) and the cartilage matrix displays phosphatase activity in that region.

The parachordals in the region of the myodome between the pro-otic and the basi-occipital are negative. The anterior tip of the notochord is strongly positive and a little farther back the central part of the parachordals also displays phosphatase activity, first in the nuclei only, then throughout the matrix. The space between the parachordals contains strongly positive connective tissue (Text-fig. 6). This, according to Schleip, ossifies later. The basi-occipital is now seen in the form of two perichondral lamellae round the



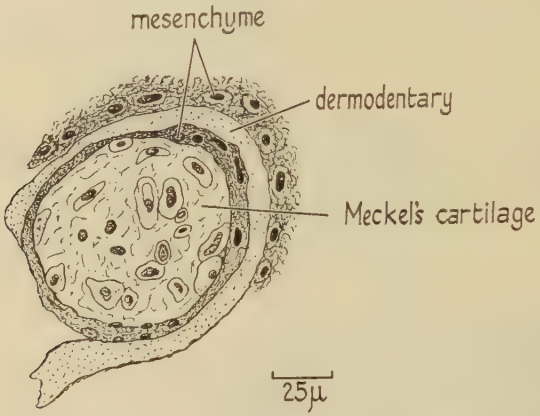
TEXT-FIG. 7. From the lower jaw of a 22-mm. trout. Decalcified section. Incubation time 15 hours. Anterior tip of Meckel's cartilage and the autodentary. The cartilage is strongly positive in nuclei and matrix. The bone is negative.

central ends of the parachordals (Text-fig. 6). Still farther back the parachordals are adjacent to the notochord and the bony lamellae continuous with the notochord sheath.

The splanchnocranium of this stage shows very clearly the correlation between perichondral bone and *extracellular* phosphatase and the absence of any correlation between (*a*) perichondral bone and *nuclear* phosphatase, and (*b*) membrane bone and phosphatase within adjacent cartilages. The reaction in the matrix is never as intense as the reaction given by the enlarged chondrocytes in the ossifying zone.

The appearance of Meckel's cartilage is as described for the previous stage. Teeth at various degrees of development are present. The mesenchyme round the tooth buds and beneath the mouth epithelium is strongly positive (Text-fig. 7). As the autodentary decreases in thickness posteriorly, the concentration of phosphatase in Meckel's cartilage gets less as already

described for the younger specimens. In Text-fig. 8 there is a thin layer of mesenchyme between cartilage and bone, and the extracellular phosphatase in the former has completely disappeared. The nuclei are still positive but the intensity of this reaction diminishes and finally the cartilage is completely negative in its distal part, although the dentary approaches it again. But there is always some mesenchyme between the two structures. Thus it is seen again that mere proximity of bone is not correlated with a positive phosphatase reaction in the cartilage, whereas actual apposition of bone is associated with a marked staining of chondrocytes as well as matrix.



Text-fig. 8. From the lower jaw of a 22-mm. trout. Decalcified section. Incubation time 15 hours. Shows a more distal region of Meckel's cartilage than Text-fig. 7. The dentary is here separated from the cartilage by connective tissue. The cartilage matrix is negative. In Text-figs. 7 and 8 the bone itself shows no phosphatase activity.

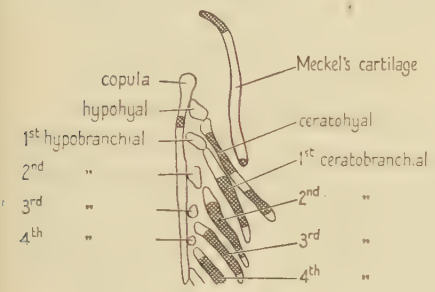
This is also illustrated by the pterygoquadrate and the hyomandibula. Ossification in the other branchial cartilages has made marked progress since the previous stage examined. The distribution of extracellular phosphatase and perichondral bone is shown in Text-fig. 9. The two perichondral ossifications of the ceratohyal cartilage have increased in thickness and are heavily calcified. The ceratobranchials display phosphatase activity and perichondral bone in their central portions only. Their anterior and posterior tips remain negative. Hence the appearance in transverse sections varies according to the level. The hypobranchials are negative throughout and have no osteoid or bone. Nuclear phosphatase is present in most of the visceral cartilages and the perichondrium is positive in some regions especially on the dorsal surface of the copula where the dermentoglossum (membrane bone) is now developing ventrally to a set of teeth to which it becomes attached. The mesenchyme in this zone is strongly positive as has already been pointed out at earlier stages. A detailed description of the chondrocranium is given in my thesis (Lorch, 1948).

Membrane Bone. A number of new membrane bones have now taken shape and some of the bones previously described are beginning to assume the

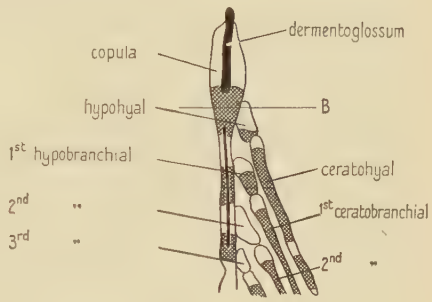
appearance of a meshwork of ground substance interspersed with cells which are penetrating from the surrounding mesenchyme. The newly formed bones, whether they arise in connexion with the lateral line canals such as the nasals and frontals, or as flat plates to be fused later with teeth such as the vomer and pre-maxilla, do not differ from the description given for developing membrane bones of earlier stages.

Summary of Stage 4

There is little or no phosphatase in non-skeletal tissues with the exception of the central nervous system and some sensory organs which remain strongly positive. All ossifying cartilages display nuclear as well as extracellular phosphatase, while the matrix of non-ossifying cartilages is always negative. The perichondrium of most cartilages displays enzyme activity. The phosphatase content of a cartilage is in no way correlated with the proximity of a membrane bone. The latter are positive at the growing-points.



TEXT-FIG. 9



TEXT-FIG. 10

TEXT-FIG. 9. Diagram of the principal visceral cartilages at the 23-mm. stage based on serial sections showing phosphatase distribution. (Modified after de Beer's reconstruction of a 16-mm. embryo.) Dorsal view of right side. Areas of cartilage bone formation and extracellular phosphatase are shaded.

TEXT-FIG. 10. Diagram of the principal visceral cartilages of a 38-mm. trout. Shading indicates extracellular phosphatase. Membrane bone is shown black. The level of the section shown in Pl. 1, fig. 6, is indicated by the line B.

Stage 5 (29–38 mm.)

From the point of view of phosphatase distribution the change from the 23-mm. embryo to the relatively mature state reached at 38 mm. is so gradual that it is best to describe the appearance of the oldest specimens so as to bring out major developments.

The following account is based on serial sections through a 38-mm. trout (about 3 months after hatching), which was decalcified for 5 hours. Sections were incubated for 6 or 16 hours, and counterstained with eosin. A 36-mm. specimen, cut undecalcified, served for comparison, especially of the calcified structures, but was found histologically inferior owing to the difficulty of cutting thin serial sections through such brittle material. With regard to the somewhat smaller specimens, fairly good sections were obtained and the

two-colour technique, using gallamine blue for phosphatase, was found most useful, in that the sites both of calcification and of phosphatase activity could be visualized in the same section.

A. *General Distribution of Phosphatase*

The general distribution of phosphatase in non-skeletal tissues is mainly as described for Stage 4. The mesenchyme has changed somewhat in appearance in the 38-mm. specimens: fibres have developed between the cells and the latter may now be called connective tissue cells rather than undifferentiated mesenchyme. It has previously been noted that phosphatase in the mesenchyme was at first widespread but then tended to become localized in the regions of membrane bone or tooth development. There seems to be a slight reversal of this tendency: areas of connective tissue not in immediate contact with calcifying structures are found to be strongly positive in the cells—many of which have processes—and in the fibres. Since there is an extensive formation of fibres at this stage the reappearance of phosphatase in the connective tissue may be related to collagen formation.

B. *Skeletal Tissues*

The classification of cartilage described for Stage 4 applies here equally.

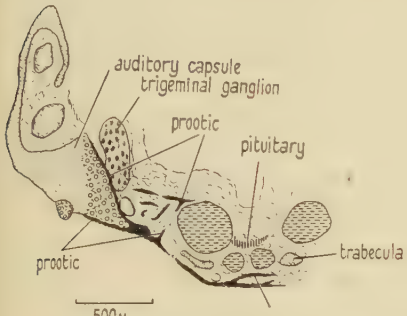
The trabecula communis and the paired trabeculae are negative. The anterior parts of the parachordals remain strongly positive in the region of the pro-otic. This bone has developed extensively and comprises perichondral lamellae of the anterior end of the parachordals, the anterior wall of the auditory capsules, the roof of the myodome, and the lateral commissure. The inner lamella of the pro-otic lies below the cerebrum and the outer represents ossification of the major part of the base of the skull.

The development of the pro-otic is a typical example of perichondral and endochondral ossification with participation of ossifying connective tissue and will therefore be described in some detail.

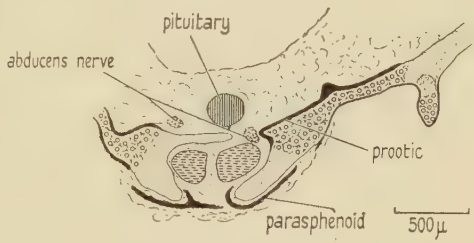
With regard to the distribution of phosphatase in the region of the pro-otic, the same principles hold as for cartilage bone formation elsewhere in so far as the pro-otic is represented by perichondral lamellae. Where it is formed by ossification of the membranes of the brain, the latter display strong phosphatase activity. The bone itself—whether perichondral or membranous —only displays very slight phosphatase activity at the edges. Examples of transverse sections through the region of the pro-otic will show its complicated structure and its relationship to cartilage, membranes, and to the parasphenoid bone: Text-fig. 11 shows the left half of the anterior region of the myodome and the exit of the trigeminal nerve. The pro-otic is seen as a perichondral lamella of the anterior auditory capsule and extending inwards towards the parasphenoid. Above this extension, the pro-otic has formed a network of interlacing trabeculae connecting dorsally with the ossified membranous base of the brain and forming the lateral wall of the myodome. The nuclei of the cells filling the spaces between the bony

trabeculae are positive, as are also the nuclei of all other tissues in the region shown: ganglion cells of the trigeminal nerve nucleus, Schwann cells, nuclei of striated muscle, connective tissue cells, and erythrocytes. The only marked concentration of extracellular phosphatase is seen in the ossifying cartilage.

Slightly distal to the level shown in Text-fig. 11, the lateral commissure becomes visible. It is strongly positive in its middle portion where it is covered dorsally and ventrally by perichondral lamellae of the pro-otic. But its central tip is negative. The parasphenoid extends below this part of the lateral commissure but is separated from it by the perichondrium.



Text-fig. 11



Text-fig. 12

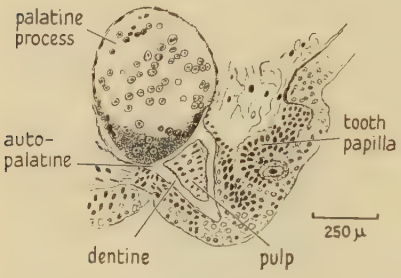
Text-figs. 11 and 12. Diagrammatic cross-sections through the posterior myodome region of a 38-mm. trout to show the distribution of phosphatase in the cartilage and its relation to associated bones. Key to shading as in Text-fig. 3. Description in text.

In Text-fig. 12 the cartilaginous roof of the myodome is seen. Its cells are arranged in transverse rows. At the point shown only the perichondrium and the chondrocyte nuclei have phosphatase activity. But distal to this region, where the pro-otic grows over the cartilage, the latter is strongly positive. The entry of the abducens nerve into the myodome is seen. The lateral walls of the myodome are formed by the anterior ends of the parachordals. Ventrally the parasphenoid is seen to curve inwards and partially surrounds the parachordals. The latter are positive only where the pro-otic has formed a perichondral lamella.

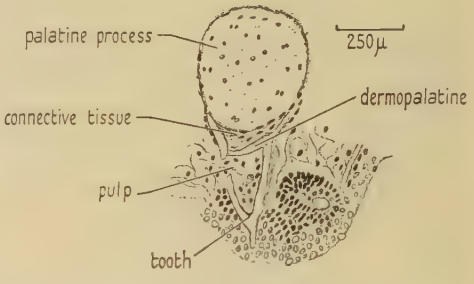
The extreme tip of the notochord is strongly positive and surrounded by an ossified sheath which is continuous with the basi-occipital. More distally only the notochord sheath contains phosphatase, the central tissue being negative. The basi-occipital forms perichondral lamellae dorsally and ventrally to the parachordals which are now positive. The ventral wall of the posterior semicircular canal is negative but becomes positive laterally where the exoccipital bone is developing. A bony lamella lying in the strongly positive membranes covering the ventrolateral aspect of the brain connects the basi-occipital with the roof of the brain. The exoccipital is well developed round the jugular foramen and the cartilage in that region is positive.

Splanchnocranium. Calcification of the autodentary although intense in places is not complete. The growing-surfaces are free from calcium salts. The bone and osteoid are relatively poor in phosphatase, but the connective tissue surrounding the dentary is strongly positive especially at the growing-tips. The phosphatase seems to be mainly nuclear, the cytoplasm of the osteoblasts and connective tissue cells is negative. But in areas of very great phosphatase activity some positive fibrils are seen.

The middle portion of Meckel's cartilage contains no extracellular phosphatase in these older specimens but the chondrocyte nuclei and the perichondral fibroblasts are positive in some areas. In the oldest fish examined



Text-fig. 13



Text-fig. 14

Text-figs. 13 and 14. From decalcified sections through a 38-mm. trout. Incubation time 6 hours. The palatine process of the pterygoquadrate is shown at two levels. In Fig. 13 the palatine bone forms a perichondral lamella and the ventral region of the cartilage is positive. In Fig. 14 the palatine bone is separated from the cartilage which has only nuclear phosphatase.

(36–8 mm.) Meckel's cartilage shows two regions of phosphatase activity in its posterior portion: just before articulation with the quadrate there is a patch of positive cartilage which at first sight seems to have no relation to any perichondral bone since it is mainly in the centre of the cartilage. But closer inspection of consecutive sections shows that in some places the angular does come into direct contact with the cartilage laterally. The angular, according to Haines (1937), has taken the place of the articular in most teleosts. Like the dentary it has a cartilage and a membrane bone portion, the former being regarded by previous authors as the articular (autoarticular of Böker).

The Pterygoquadrate. At all previous stages the palatine process of the pterygoquadrate was completely negative or only showed slight phosphatase activity in nuclei and perichondrium. The palatine bone was then separated from the cartilage by a thin layer of connective tissue. In the 38-mm. specimen a different picture is obtained: anteriorly the palatine is composed of a very thin perichondral lamella and a membrane bone portion to which the teeth are fused. The two parts of the bone are partially separated by a very thin layer of connective tissue but are fused laterally. The cartilage is positive at its ventral edge, i.e. where the autopalatine is in apposition (Text-fig. 13). The dermopalatine extends farther backwards than the autopalatine. Hence

more distal sections show no perichondral lamella and also no phosphatase in the cartilage (Text-fig. 14). The dermopalatine is separated from the palatine process by a thin layer of connective tissue, the nuclei of which are positive. In the pterygoid region the cartilage is negative and the perichondrium positive, being in close relation to the two membrane bones (ecto- and endopterygoid) of that zone.

The distribution of phosphatase in the quadrate is similar to that at the previous stage. The tendinous tissue connecting the quadrate with the symplectic is strongly positive. It is said to ossify.

The symplectic bone is becoming thicker at the expense of the central cartilage which is very strongly positive and shows much-enlarged cells with degenerating nuclei. There is resorption of cartilage and formation of marrow spaces near the foramen for the hyomandibular branch of the facial nerve. The cartilage in that region is strongly positive. The nuclei of the perichondral and periosteal cells show phosphatase activity; Schwann cell nuclei as well as those of bone-marrow cells are also positive. In all the branchial cartilages where perichondral bone is formed there are well-defined zones of extracellular phosphatase. Areas free from bone have no phosphatase. The degree of phosphatase activity does not seem to depend on the thickness of the perichondral bone. Text-fig. 10 is a diagram of part of the branchial skeleton showing the position of 'positive cartilage' in a 38-mm. trout. This is based on serial sections, an example of which is given in Pl. 1, fig. 6. With regard to the two membrane bones dorsal to the copula the following point is again illustrated: there is no correlation between a positive reaction of the cartilage and the proximity of a membrane bone.

Membrane Bone. In the oldest specimens examined, the histological structure of the membrane bones is now clearly that of an 'adult' trout. There is no sharp transition to adult condition such as is found, for instance, in mammalian long bones where the epiphyseal cartilage is replaced by bone. Since fish continue growing throughout their life if conditions are favourable, the term 'adult' must be applied with reservations. The chief difference between teleost bone and mammalian bone lies in the relative scarcity of osteocytes. According to Kölliker (1859) these are entirely missing in some teleosts. He called the acellular tissue 'osteoid', a term which I have used here to describe *uncalcified* bone irrespective of the presence of osteocytes. Schmid-Monnard (1883) admits that the primary bony lamella is a structureless acellular mass, but in adult bones osteocytes are occasionally seen. Stéphan (1900) points out that both acellular and cellular bone is found in teleosts and that the former invariably consists of thin lamellae through which nutrients could diffuse, thus eliminating the necessity for a vascular system such as the Haversian systems of mammalian compact bone. The last observation is confirmed by the present series: thin, bony lamellae—whether perichondral or membrane bone—are devoid of cells. Many membrane bones, e.g. the nasals and frontals, remain thin plates and only very few osteocytes could be found in such bones. But bones which rapidly increase in bulk such

as the pre-maxillae and maxillae and the perichondral ossification of the symplectic show a fair number of cells within the ground substance (Pl. 2, fig. 1). Marrow spaces are seen in some bones. The ground substance shows lines separating the older from the more recently secreted matrix. Near the outer limit of the bone there is often a black line indicating phosphatase activity. Osteoblasts, if present, are ranged outside the osteoid layer and are usually strongly positive, as for instance at the distal end of the maxilla (Pl. 2, fig. 4). The same relationship as has just been described for bone, osteoid, and osteoblasts exists between calcified dentine, uncalcified dentine, and odontoblasts.

The relationship between membrane bone and cartilage at this stage is illustrated by the vomer in the region where it forms a continuous lamella roughly following the shape of the cartilage from which it is separated by a layer of very cellular, intensely positive connective tissue (Pl. 2, figs. 2 and 3). Below this bony lamella the connective tissue is less cellular, but the nuclei also display strong phosphatase activity.

The frontal may be mentioned here as a typical 'canal bone'. It consists of a bony tube surrounding a lateral line canal and a flat lamella extending inwards towards the mid-dorsal line. Another shorter process extends outwards from the canal. The bony plates are separated from the cartilage of the tectum cranii by a very thin layer of tissue which is both perichondrium and periosteum. It is never thicker than three layers of fibroblasts. As has constantly been noted for connective tissue between cartilage and bone, it has marked phosphatase activity. The underlying cartilage is negative. The frontal bone itself is negative in its thicker (peripheral) portions. It tapers to a thin end centrally and here, i.e. at the growing-point, it displays the 'positive lines'. The walls of the tube surrounding the lateral line canal are somewhat thicker than the flat part of the frontal and occasional osteocytes are seen. Again the growing (dorsal) tips of the bone have some phosphatase peripherally and accumulations of osteoblasts are seen. These are strongly positive, but so are most other connective tissue cells and fibres. Groups of the large round cells, noted also in connexion with the pre-opercular, are seen at the junction of the canal bone portions and the flat part of the frontal. These cells are devoid of phosphatase except for the nucleoli which stain faintly. Minute fragments of bone are sometimes found between them, and these fragments, unlike the newly formed osteoid, are free from phosphatase. The large cells suggest a possible osteolytic function. Although the connective tissue between bone and skin epithelium is strongly positive, the epithelial cells themselves display on the whole no phosphatase activity.

Summary of Stage 5

Further examples of the correlation between perichondral ossification and extracellular phosphatase are given. The structure of some membrane bones is described.

Discussion

The following points emerge from the study of the distribution of phosphatase and 'bone salts' in growing trout.

In the early stages of development the enzyme is widely distributed and is on the whole confined to the nuclei. As differentiation proceeds phosphatase becomes more concentrated at sites of bone or fibre formation, while the nuclei of the undifferentiated mesenchyme and non-calcifying cartilage display less phosphatase activity. With the appearance of perichondral osteoid the chondrocytes undergo a marked change in appearance and, simultaneously, phosphatase activity spreads from the cells to the matrix. The change is reminiscent of that observed in mammalian hypertrophic cartilage. It is significant that bone is never formed in the absence of extracellular phosphatase. This observation is in agreement with that previously made on mammalian bones (Lorch, 1947). It must be noted that the maximum concentration of phosphatase in the cartilage occurs before there is any sign of calcification and at sites which do not themselves calcify. The pre-osseous substance seems to contain very little phosphatase. However, the perichondrium (which becomes the periosteum) is always strongly positive. A speculation regarding the source of phosphatase in cartilage bone formation is of interest. In mammalian endochondral ossification the enzyme is said to be derived from osteoblasts as well as hypertrophic cartilage cells. In the trout, osteoblasts are not prominent and are especially rare in connexion with perichondral bone formation. Therefore the most likely sources of phosphatase are the enlarged cartilage cells which may secrete the enzyme into the matrix with which the bone is in contact. The strongly positive reaction of these cells and the adjacent matrix favours this view. The fact that the cartilage itself does not calcify at the stages examined is surprising, but may be a necessary condition for the diffusion of the enzyme from the cells to the periphery.

In membrane bone formation increased phosphatase activity was also noted in the mesenchyme well before the onset of calcification. The enzyme occurred in fibres as well as cells, but the change from purely intracellular to extracellular phosphatase was not as marked here as in cartilage bone formation.

However close a membrane bone is to a cartilage, the latter never displays phosphatase in the matrix, unless it is itself ossifying. Hence the two types of bone can easily be distinguished by means of the Gomori method.

The absence of phosphatase from the calcifying osteoid and the fact that calcification starts at the centre, whereas the highest concentration of enzyme is found at the periphery, may seem surprising. However, if it be considered that osteoid is a dense avascular tissue surrounded on all sides by a zone of high phosphatase activity, it seems likely that the inorganic phosphate liberated at the periphery tends to accumulate within the osteoid and so a high level of phosphate ions may be reached without the presence of phosphatase at the actual site of calcification.

As calcification proceeds, new layers of osteoid are formed at the periphery. Once the bone is well defined, its phosphatase content is usually very low, except at the growing-tips and edges. Very few cells are enclosed in the bony matrix and they contain no phosphatase. The phosphatase content of the mesenchymal cells and fibres stays high as ossification proceeds although the bone itself may be quite negative.

The biochemical studies of phosphatase in fish, mentioned in the introduction, must necessarily deal with organs or parts of organs and no information regarding the distribution of phosphatase within a tissue or its intracellular distribution can be gained from them. The facts previously established may be briefly reviewed in the light of the present work: Roche and Bullinger (1939) found that 'phosphatase was present in all teleost bones examined and high concentrations of enzyme were present in scales and teeth'.

Roche and Collet (1940), working on the sardine, found that there was a a seasonal increase in phosphatase activity in the *whole* skeleton during spring and early summer, i.e. when optimum conditions for growth prevail. They emphasize that this constitutes evidence for the physiological regulation of phosphatase activity in the skeleton as a whole, possibly by an endocrine mechanism. Without wishing to contradict this hypothesis for which there is independent evidence (Roche and Filippi, 1938), I think it should be pointed out that the increase of phosphatase activity in different parts of the skeleton may be 'simultaneous' when reckoned in terms of months, but histochemical studies of the ossification of the chondrocranium of the trout show that the increase of phosphatase activity of each cartilage—or portion of cartilage—is exactly correlated with the appearance of perichondral osteoid round the particular cartilage and in no way influenced by ossification in an adjacent cartilage or within the mesenchyme.

With regard to the distribution of phosphatase in the embryo generally a comparison with results obtained by Moog (1944) on chick embryos and by Horowitz (1942) on the heads of foetal rats is interesting.

According to Moog 'phosphatase persists as long as a tissue remains undifferentiated. As differentiation proceeds, phosphatase in some cases disappears and in others accumulates in higher concentrations than in the primitive phase.' Although the trout embryos examined did not include the very early stages of development comparable to Moog's chick embryos, her statement is on the whole confirmed and similar observations were made on the marine teleost *Cottus bubalis*, early stages of which were examined as a preliminary study (unpublished) to the present work.

Horowitz (1942) commences his study of phosphatase and glycogen with rat foetuses at the gill arch stage (13 days) and finds them 'devoid of phosphatase'. However, incubation was only carried out for 2 hours.

In 15-day foetuses Horowitz notes that prospective regions of calcification show a marked phosphatase activity, i.e. they become chemically differentiated before the occurrence of any morphological differentiation. This is in accord with the present results. Also the irregular distribution of phosphatase in the

central nervous system, and its high concentration in the linings of blood-vessels, in taste-buds, and in the lens epithelium are paralleled in the trout embryos.

With regard to ossifying cartilage Horowitz's results are in agreement with previous descriptions for other species: hypertrophic cartilage containing high concentrations of phosphatase in cells, matrix, and perichondrium is invariably associated with ossification.

It is seen that apart from minor differences, there is a striking parallelism between phosphatase distribution in developing embryos belonging to species as widely different as chicks, rats, and trout. It is therefore indeed likely, as suggested by Moog, that phosphatase plays a fundamental role in histogenesis, apart from its function in the development of calcified structures.

I should like to thank all those who have helped me by their advice and criticism, particularly Dr. J. F. Danielli for his encouragement throughout this work.

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Summary

1. The histological and cytological distribution of alkaline phosphatase in developing trout has been studied with special reference to membrane and cartilage bone formation in the skull.

2. Nuclear phosphatase is widely distributed in the youngest stages examined, but decreases as differentiation proceeds.

3. Extracellular phosphatase is always associated with ossification or fibre formation.

4. No deposition of calcium salts in the absence of phosphatase was observed.

5. Alkaline phosphatase is probably connected with histogenesis in general apart from its special function in calcification.

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DESCRIPTION OF PLATES 1 AND 2

All figures are unretouched photomicrographs of undecalcified sections treated by the Gomori (1939) method, unless otherwise stated. Areas of phosphatase activity are shown black. No counterstain was used.

PLATE 1

Fig. 1. Trabecula communis of a 12-mm. trout. Incubation time 6 hours. The chondrocyte nuclei are positive. Note the strong reaction of the mesenchyme dorsal to the trabecula.

Fig. 2. Quadrate and hyosymplectic cartilage of a 12-mm. trout. Incubation time 6 hours. Note the strongly staining mesenchyme lateral to the cartilages. This is the site of the future pre-opercular bone. Chondrocyte nuclei and perichondrium are positive.

Fig. 3. Transverse section through the anterior auditory region of a 20-mm. trout. Incubation time 2 hours. Note the intense reaction of the ceratohyal and the absence of phosphatase from the parachordals and auditory capsule. The hyomandibula shows patches of phosphatase activity.

Fig. 4. Quadrate process of a 20-mm. trout showing perichondral bone. Incubation time 2 hours. Note the strongly positive reaction of the matrix near the zone of ossification.

Fig. 5. Distal end of quadrate cartilage at its articulation with the symplectic process. 20-mm. trout. Incubation time 6 hours. Note the difference in appearance and phosphatase content between non-ossifying and ossifying cartilage: only the symplectic is surrounded by a layer of osteoid.

Fig. 6. Copula and hypohyals of a 38-mm. trout. Level B in Text-fig. 10. Decalcified transverse section, incubation time 6 hours. Counterstained with eosin. Note the positive reaction of the copula which has a shell of perichondral bone. The hypohyals are only positive ventrally where ossification is beginning.

PLATE 2

Fig. 1. Undecalcified premaxilla of a 30-mm. trout. Bone salts visualized as cobalt sulphide. *Not* incubated for phosphatase visualization. Note cell spaces in the bone.

Fig. 2. Trabecula communis and vomer of a 32-mm. trout. Incubation time 15 hours. Note the strongly positive connective tissue in the area of tooth formation.

Fig. 3. Detail from centre of previous figure. Note the clear zone of osteoid (free from both calcium salts and phosphatase) on both sides of the vomer. The perichondrium on the ventral aspect of the trabecula is strongly positive.

Fig. 4. Distal end of maxilla of a 29-mm. trout. Incubation time 4 hours. The maxilla is not calcified in this zone. It displays faint phosphatase activity. Osteoblasts are seen on both sides of the osteoid. The surrounding connective tissue is strongly positive.

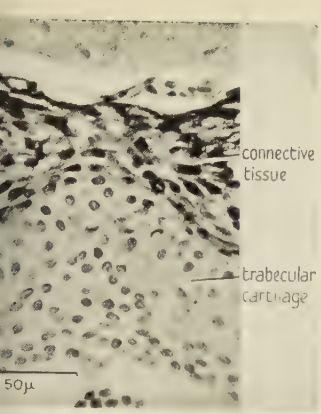

FIG. 1

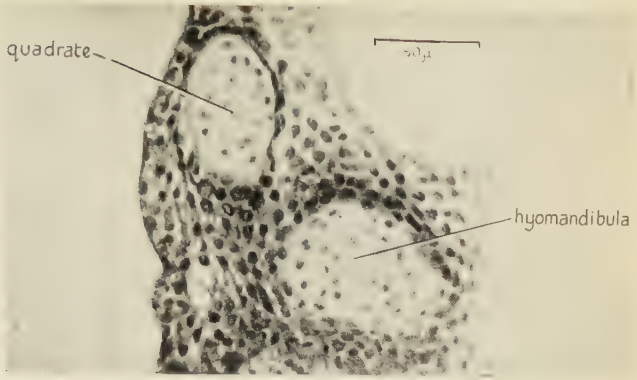

FIG. 2

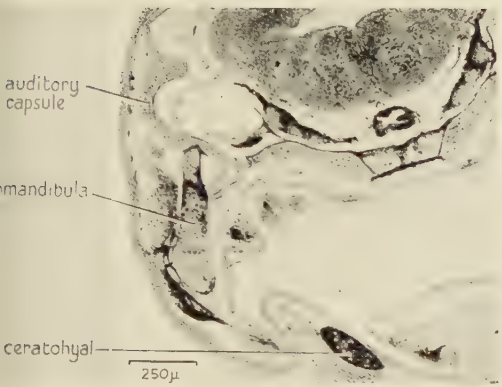

FIG. 3

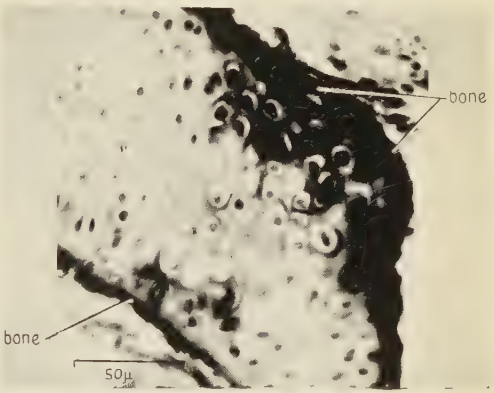

FIG. 4

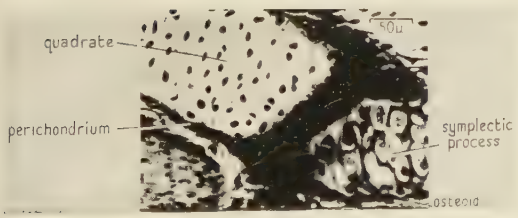

FIG. 5

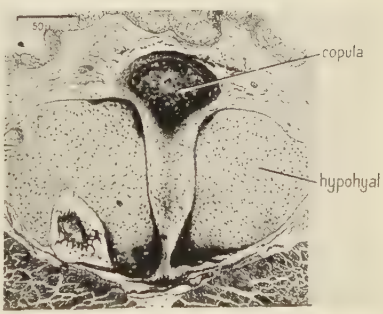

FIG. 6

J. LORCH—PLATE I

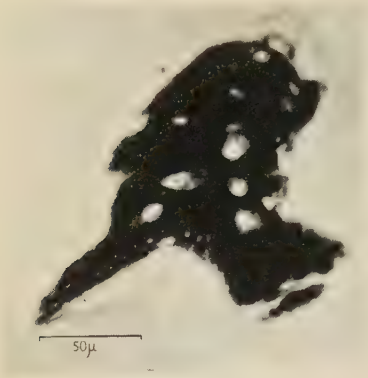

FIG. 1

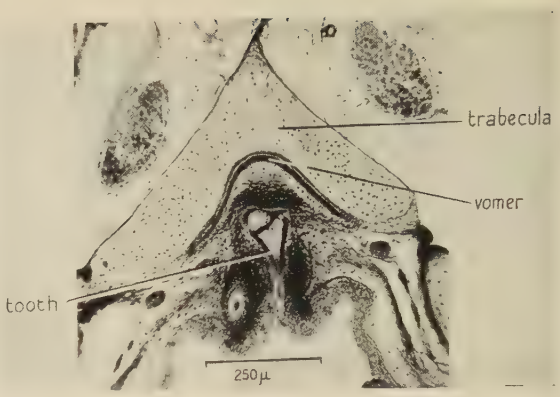

FIG. 2

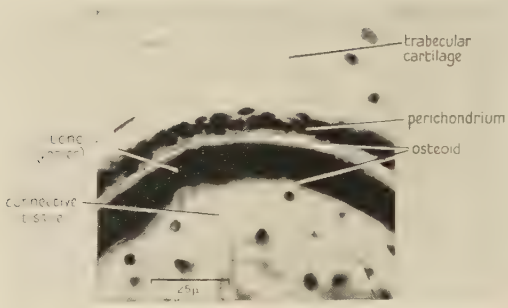

FIG. 3

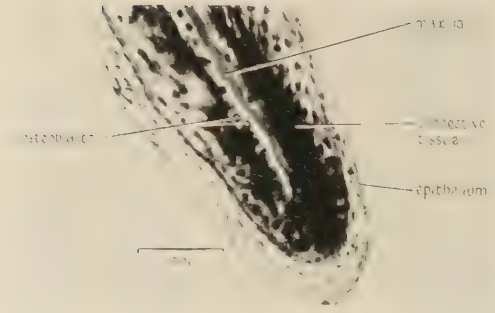

FIG. 4

J. LORCH—PLATE II

Location of Absorbed Carcinogens within the Amphibian Cell

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Introduction

SOME of the polycyclic hydrocarbons derived from phenanthrene are biologically active in a number of different ways. Not only are they, as is well known, capable of causing the production of cancers and simulating the action of sex hormones, but they can also evocate neural tissue in the amphibian embryo (Waddington and D. M. Needham, 1935), and exert a rather feeble mutagenic activity (Demerec, 1947, 1948; Strong, 1947; Carr, 1947).

In none of these connexions have we a clear idea of the mechanism by which the effect is produced. This paper is, in the first instance, concerned to throw further light on the interaction between the carcinogenic substances and the embryonic amphibian cells on which they exhibit the activity of evocation. There are several questions which present themselves in this connexion. One is the relation between the carcinogen evocators and the substance, which may be called the 'natural evocator', which is presumably responsible for stimulating the ectoderm to differentiate into neural tissue during normal development. The demonstration of Waddington, Needham, and Brachet (1936) that evocation could be produced by treatment with methylene blue proved that evocation can be brought about by substances which are certainly not present in normal eggs. Waddington, Needham, and Brachet pointed out that such substances might act by killing a few of the neighbouring cells, thus releasing their stores of the natural evocator, which stimulates the surviving cells to neural differentiation. Holtfreter (1944*b*, 1945) and others following him at first suggested that all evocations by chemical implants were mediated by this mechanism. But recently Holtfreter (1948) appears to have convinced himself of the truth of the contention of Waddington, Needham, and Brachet that evocation is possible even when no signs of necrosis can be detected; and he seems ready to accept their suggestion that non-natural evocators may act, in some manner less drastic than killing, so as to release a store of natural evocator which is contained within

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the ectoderm cells and which then brings about an induction. It is very possible that the carcinogenic hydrocarbons owe their evocating power to an action of this kind. Alternatively, they might be related chemically to the natural evocator, about whose properties we are still completely in the dark. Waddington (1940) and Needham (1942) have suggested that the low dosage in which the evocating activity is manifested offers some support for this suggestion; but they admit that too much weight cannot be placed on the argument.

It was not to be expected that, from observations of the kind to be recorded in this paper, any direct evidence would be forthcoming on the general question of whether the carcinogens acted as substitutes for the natural evocator or in a secondary way as releasers of it. But it was anticipated that some light might be thrown on their relation to one of the other substances which have been claimed to be the natural evocator. Fischer, Wehmeier, and Jühling (1933) first showed that preparations of ribonucleic acid can act as evocators, but they did not exclude the possibility that this was a result of producing localized necrosis. Brachet (references and discussion in 1947) has confirmed the result, using purified preparations; and has also presented evidence to show that when dead tissue is digested with ribonuclease, it loses its evocating power. He argues that the synthesis of cell-protein is carried out mainly at the ultra-centrifugeable ribonucleoprotein granules in the cytoplasm, to which Claude (1941) had first directed attention; and he suggests that evocation is primarily a reaction which takes place at these granules. He appears to believe that the natural evocator substance is actually ribonucleic acid, which diffuses from the roof of the primitive gut into the overlying ectoderm and leads to an increase in concentration there of these cytoplasmic granules. And he further suggests that the activity of the carcinogens is due to their property of becoming attached to the granules.

In making the last suggestion, Brachet bases himself on the observations of Graffi (1939, 1940). This author studied the accumulation of various polycyclic hydrocarbons within the cells of normal and malignant tissues of the mouse. The technique used was to render the hydrocarbons water soluble by treatment with glycerine and blood-serum, to expose the cells to aqueous solutions containing up to about one part of hydrocarbon in 40,000, and to study the location of the chemical within the cell by observing the fluorescence of the cell constituents when illuminated with ultraviolet light. Graffi found that the hydrocarbons become attached to cytoplasmic granules. He speaks of these as 'lipochondria' and 'mitochondria', but it is notoriously difficult to be certain exactly what various authors mean by these terms; and it was perhaps not out of the question that Brachet might have been correct in suggesting that some of the granules which accumulate the substances may be identical with the ribonucleoprotein granules (which may be called 'microsomes') to which he attributes synthetic activity.

Although Brachet's theory provides an attractive way of envisaging the mode of action of hydrocarbons in both carcinogenesis and evocation, it is

clear that its observational basis is rather slender. We have therefore used methods essentially similar to Graffi's to investigate the accumulation of hydrocarbons in the cells of the amphibian embryo. This material has the advantage not only that it is the tissue on which the evocatory action is exerted, but that its microsomes have been described by Brachet, while its cytoplasmic lipoids have been fully studied by Holtfreter (1946*a*, *b*, *c*).

Apart from the somewhat involved questions relating to Brachet's theory of evocation, a much simpler problem remains to be solved and it was hoped that the present investigations would throw some light on it. It has been apparent for some years that the cell surface plays an extremely important part in many of the morphological changes occurring during development and in particular during the formation of the neural tube (Waddington, 1942; Holtfreter, 1943, 1944*b*). It might with some plausibility be suggested that the carcinogenic hydrocarbons owe their evocating power to their well-known surface activity (Moricard and Gothié, 1943*a*, *b*, 1944). If this were the case, one would expect to find them localized in the surface membrane of the cell.

Technique

Explants of the blastocoele roof of early gastrulae of the newt *Triturus alpestris* were cultured overnight in Holtfreter's standard solution containing solubilized 3:4-benzpyrene. They were then examined microscopically by ultra-violet light which causes those structures in the cell which have accumulated any of the hydrocarbon from the culture medium to show a bright blue fluorescence. The light source was an Osram high-intensity lamp with a quartz lens and filter to exclude all visible light. At first a surface-silvered mirror and quartz condenser were used on the microscope, but it was later found that the standard glass mirror and condenser gave equally good results.

Weil-Malherbe (1946) has shown that various polycyclic hydrocarbons including 3:4-benzpyrene will form water-soluble addition-compounds with caffeine and other purines. In the present investigations this method was used for rendering the benzpyrene soluble, in preference to the more complicated way of using saturated solutions in hot glycerine diluted with serum, adopted by Graffi. A 1 per cent. solution of caffeine in Holtfreter's standard solution was stirred overnight with a slight excess of benzpyrene at 20° C. and then filtered. The resulting solution, which shows distinct purplish-blue fluorescence in sunlight should, according to the figures given by Weil-Malherbe, contain about one part in 100,000 of dissolved benzpyrene. In one series of experiments this solution was diluted ten times again with Holtfreter's solution, which should give about one part in 10,000,000 of the benzpyrene. The cells of the explant cultured in this diluted solution were nearly as brightly fluorescent in ultra-violet light as were those in the stronger one and even in a solution diluted by a further factor of 10 the cells still showed a very faint bluish fluorescence. Amphibian gastrula cells cultured

in pure Holtfreter's solution or in a 1 per cent. solution of caffeine without any benzpyrene show practically no fluorescence, at the most only the very faintest greenish, not blue, tinge.

The cells of the explants in 1 per cent. caffeine in Holtfreter's solution, both with and without dissolved benzpyrene, tend to dissociate from one another and fall apart in the same way as when they are cultured in an alkaline medium. This happens also in a 1 per cent. caffeine solution buffered to pH 6·9. The dissociated cells appear otherwise to be quite healthy and remain alive for several days, although they are apparently not able to re-aggregate when transferred to a caffeine-free solution as happens when cells dissociated by alkali treatment are returned to a neutral medium (Holtfreter, 1947). Explants cultured in 0·1 per cent. caffeine with or without added benzpyrene show little or no tendency to fall apart. No cytological differences could be observed between the dissociated and undissociated cells, and, as the former in the higher concentration of benzpyrene showed a rather brighter fluorescence in ultra-violet light, they were used for most of the observations.

A few observations were made with other types of cells, particularly to search for any evidence that fluorescent compounds were accumulated in the nucleolus (see p. 215).

Experimental Results

After having been cultured for some time in a benzpyrene-caffeine solution, the cells of the explant will have fallen apart and become spherical with a diameter of about 60μ, though there is considerable variation in size. Seen by ultra-violet light they show the characteristic blue fluorescence of benzpyrene in molecular solution, though otherwise it is hard to distinguish much structure in the spherical cell. The nucleus is seldom visible as it is obscured by the overlying fluorescent cytoplasm. When the cell is squashed slightly with a coverslip more of its structure becomes visible. The nucleus, as reported by Graffi, is entirely non-fluorescent and shows up as a dark patch in the middle of the cell. The fluorescent nucleolus described by Graffi as being occasionally visible in mouse cells was not observed in this amphibian material. Rarely a bright fluorescent liposome was seen outlined against the dark nucleus, which might have been mistaken for a nucleolus, but these were always outside the nuclear membrane.

Neither the cell membrane nor the nuclear membrane showed any fluorescence. This is true both of the intercellular membrane and of the 'surface coat' (Holtfreter, 1943, 1944*a*) which forms the boundary of the egg against the external medium. When a cell or group of cells is partly covered by surface coat containing pigment this material can be seen as a dark patch, itself not fluorescent, obscuring the light coming from the underlying cytoplasm. The cytoplasm showed a uniform bright blue fluorescence, though not infrequently the nucleus was outlined by a ring or crescent considerably brighter than the rest of the cytoplasm. This ring, which is usually rather homogeneous and does not contain yolk platelets, probably consists of the phospholipids

associated with the nucleus which, after fixation, constitute the so-called 'Golgi Apparatus'. It could sometimes be seen in quite fresh and unsquashed cells and was probably not an artifact. The rest of the ctyoplasm in these early gastrula cells is closely packed with yolk platelets round which the lipochondria cluster in the way described and figured by Holtfreter (1946*a*). The lipochondria are brightly fluorescent and outline the considerably less bright yolk platelets. Scattered throughout the cytoplasm there are also a number of very much smaller brightly fluorescent granules or globules in Brownian movement as well as a few larger and very brightly fluorescent globules; both of these are considered to be liposomes. When the cells are squashed strongly so as to burst, or begin to dry up, and probably also simply as a result of prolonged exposure to ultra-violet light, they begin to show further changes. The lipochondria detach themselves from the yolk platelets and break up, their lipoid constituents running together to form the larger fat globules known as liposomes which are very brightly fluorescent. As the preparation deteriorates further the liposomes become larger and less numerous as they join up with one another. At the same time as the yolk platelets lose their attached lipochondria they also lose their never very bright fluorescence.

It is probable that in an entirely fresh state the gastrula cells would have most of their lipoid in lipochondria and that the liposomes appear as a result of the breakdown of the lipochondria under abnormal conditions. In cells of neurulae and later stages the liposomes do become conspicuous even in the freshest material, and they probably occur as such in the cells of the normal living embryo at those stages. In unsegmented eggs most of the lipoid is in the lipochondria associated with yolk platelets although liposomes and fat globules are also present. It is, however, impossible to examine the intracellular inclusions of an unsegmented egg *in situ*; the egg must be broken up for microscopic examination of its contents and the breaking-up process is very likely to damage some lipochondria and to release their lipide as liposomes. The contents of uncleaved eggs which had been cultured in benzpyrene-caffeine solutions were examined by ultra-violet light and their yolk platelets showed the same fluorescence picture as has been described for gastrula cells. Uncleaved eggs could not, however, be cultured whole in the benzpyrene-caffeine solution since, even when the vitelline membrane is left intact, the cell membrane and 'surface coat' disintegrate after 10 minutes or so.

Some of the explants that had been cultured in benzpyrene solution were then stained unfixed with Unna's methyl green-pyronine. In this the nucleus took up the methyl green while the cytoplasmic granules of ribonucleoprotein, or microsomes, stained red with the pyronine, as described by Brachet. By this means it is easy to distinguish between the microsomes, which are rather uniform in size just at the limit of microscopic resolution and so probably about $0{\cdot}3\mu$ in diameter, and the unstained liposomes, the smallest of which were little larger than the microsomes, but which showed a graded series in size up to the largest of 1μ or more. It is hard to distinguish between them

in unstained preparations and both are small enough to show Brownian movement. By the examination of a preparation cultured in benzpyrene solution and then stained with pyronine, first of all by visible light and then immediately afterwards changing to ultra-violet, it was possible to prove that the very small granules in Brownian movement that had taken up the benzpyrene and so fluoresced in ultra-violet light were small liposomes, while the pyronine-staining microsomes showed no fluorescence. It would therefore seem that Brachet was mistaken in assuming that the ribonucleoprotein microsomes of the amphibian gastrula are able to take up benzpyrene from the surrounding culture medium (Brachet, 1947, p. 479).

As well as the microsomes, which stain a darker red, the yolk platelets and especially their attached lipochondria stain pink with pyronine. After digestion with a solution of crude pancreatic ribonuclease the pyronine-staining properties of both lipochondria and microsomes are lost, although the microsomes still remain visible, not being dissolved away.

These observations of the location of the fluorescent material within the cell were supplemented by centrifuging experiments on single uncleaved eggs and on gastrulae ground up with Kieselguhr. In both cases, the material is sorted out (in an angle centrifuge giving about 3,500 g.) into four main zones; the lipoid accumulates at the centripetal pole, and is followed by a large, rather clear layer of cytoplasm, which is separated from the centrifugal layer of yolk granules by a thin zone of pigment and other granules. In material which had been treated before centrifugation with benzpyrene, the pigment layer did not fluoresce at all, while the fluorescence in the watery layer was also slight. The lipoid layer was extremely bright, and there was usually fairly strong fluorescence in the yolk layer, probably due mainly to lipochondria which had not been separated from the yolk granules. The main activity was certainly in the lipoid layer, as might be expected from what has been said above.

In such centrifugates, the nucleoprotein microsomes would be expected to come out at the bottom of the watery cytoplasmic layer, i.e. along with the pigment granules. No fluorescence could be found here in preparations made with a normal laboratory centrifuge. It might be argued that such an instrument would not suffice to sediment the microsomes, and that the slight luminosity of the cytoplasmic layer was due to benzpyrene absorbed on microsomes. Even if this were so, the microsomes would only account for a very small fraction of the total hydrocarbon absorbed by the cell. But the matter can be tested by further centrifugation. After a preliminary treatment in the angle centrifuge the watery layer was isolated and again centrifuged on a high-speed air-driven ultra-centrifuge of the Beams-King type. This succeeded in clearing the fluorescence; but the important point is that the granules to which the fluorescence was due moved centripetally, i.e. in the direction to be expected if they were very small fat or oil drops, but opposite to that expected if they were the microsomes discussed by Brachet.

Discussion

It is clear from the above observations that the lipoid constituents of the cell—lipochondria, liposomes, and perhaps the phospholipids constituting the 'Golgi Apparatus'—have an affinity for benzpyrene and will take it up out of solution with caffeine. There is really no evidence that the non-lipoid constituents can accumulate it.

The cell membrane, in particular, seems to absorb less of the hydrocarbon than any other part of the cell; in fact by the present technique none whatever can be detected in it. This finding makes it appear very improbable that the evocatory power of the steroid hydrocarbons is due to any influence of their surface activity on the membrane, although it of course remains possible that an activity of this kind plays an important role in their reaction with some deeper-lying structure within the cell.

There is also no direct observational evidence of any accumulation of benzpyrene by nucleoprotein structures. It is certainly not directly absorbed by the nucleoprotein microsomes with the avidity with which it is taken up by the lipochondria. This conclusion is not in conflict with the published evidence of Graffi. That author did, however, claim that the nucleolus, which consists largely of ribonucleoprotein, occasionally shows fluorescence with benzpyrene. It does not seem to us possible to be certain that the structures described and figured by Graffi really were nucleoli. He only rarely saw them, and it is hard to distinguish them with certainty from liposomes lying just above or below the nucleus. In the amphibian material no fluorescent nucleoli were detected, though again it would be difficult to identify them with certainty.

In order to test this matter on more favourable material a series of observations was made on the oocytes of the pond snail *Limnaea stagnalis*, which have very conspicuous nucleoli and comparatively little lipoid (Raven, 1948). The ovotestis was dissected out and cultured overnight in amphibian Ringer solution diluted six times, containing 1 per cent. caffeine, and saturated either with benzpyrene or with another carcinogenic hydrocarbon, 20-methylcholanthrene. The oocytes were then examined either centrifuged or gently squashed under a coverslip. By visible light the nucleolus, which is about 15μ in diameter, shows up very conspicuously; on switching over to ultra-violet light a certain amount of purplish fluorescence can be seen in the yolky cytoplasm but none at all in either the nucleolus or the nucleus. Fixed oocytes of *Echinus* kept for 24 hours in the hydrocarbon solutions also showed no trace of fluorescence in their conspicuous nucleoli. Some observations were also made on the salivary chromosomes of *Drosophila* cultured in benzpyrene-caffeine solution. If ribonucleoprotein absorbs the hydrocarbon it might have been expected that the heterochromatic regions would have been fluorescent in ultra-violet light, but no sign of this could be observed. Finally, a suspension of tobacco mosaic virus (a ribonucleoprotein), stood for some hours in solubilized 3:4-benpyrene and then centrifuged at high speed, showed no fluorescence in the centrifugate.

Weil-Malherbe (1946) detected a slight solubilizing effect on benzpyrene and other hydrocarbons by nucleotides and nucleosides *in vitro*. He suggested that this property, due presumably to their purine constituents, might be of some biological significance. It is conceivable that the ribonucleoprotein might have some slight affinity for benzpyrene but that the caffeine used as a solubilizer in this work holds on to it more strongly and so prevents its accumulation in the nucleoprotein structures. To check this point aqueous solutions of benzpyrene and methylcholanthrene were prepared according to the method described by Graffi (1939), glycerine and serum being used as the solubilizer. *Limnaea* oocytes cultured in these solutions and examined in ultra-violet light showed, however, no signs of fluorescence in the nucleolus. It must, therefore, be concluded that there is no evidence that structures containing ribonucleoproteins necessarily have any affinity for these carcinogenic hydrocarbons, whereas the evidence that the lipoid constituents readily take up the substance is direct and conclusive.

The evidence that benzpyrene is not accumulated to any significant extent by nucleoprotein structures makes it probable that the mutagenic activity of the carcinogens (Demerec, 1947, 1948; Strong, 1947; Carr, 1947) is indirect, the genetic mutations being produced as secondary consequences of a primary cytoplasmic effect. It is noteworthy that, as mutagens, these substances differ in several respects from other active chemical agents, which are thought to act directly on the chromosomes, such as mustard gas (Auerbach and Robson, 1947). Thus the increase in mutation-rate achieved with carcinogens in *Drosophila* is much less than with the mustards, and although exact dosages are known in neither case, the difference is probably real. Further it has been claimed by Strong and Carr that some carcinogens (methylcholanthrene) cause the mutation of specific genes: the effect is perhaps still open to some doubt, but nothing of the kind occurs with the mustards, and the phenomenon, if true, may prove to be characteristic of secondary mutagenic action (Hadorn, 1948).

We may now turn to consider the bearing of these observations on the way in which we envisage the mechanism of action of the hydrocarbon evocators. In the first place, the survival of amphibian embryonic cells for some considerable period in the rather strong solutions used in this work makes it unlikely that the evocation is to be attributed simply to the toxicity of the substances. Holtfreter (1945) appears to have advanced this suggestion on no better grounds than that it fitted in with his theoretical outlook. He produced no good observational evidence for it; whereas Shen (1942) noted that the explants which showed the best neuralization in solutions of 1:2:5:6—dibenzanthracene-α-β-endosuccinate were the ones which had the fewest damaged and cytolysing cells.

Sufficient attention has not always been paid to the delicacy of the balance which would have to be struck to operate the mechanism of evocation by the cytolysis of part of the exposed ectoderm. It is necessary that some cells should be actually killed, so that they release their evocator, while others

remain healthy enough to react to it by neural differentiation. It is comparatively easy to accept such an explanation in experiments where the stimulus is locally applied (e.g. by localized mechanical injury, or even by a localized implant of a relatively indiffusible chemical substance), but it is a rather less plausible explanation of the activity of an evocator which acts in solution. It then becomes necessary to suppose that some cells of the exposed ectoderm (e.g. those not protected by the 'surface coat') are much more readily accessible to the substance than the others, which are the survivors. This may in some cases be true; but the suggested mechanism has by this time become rather complicated, and there is usually no good reason to prefer it to the simpler supposition that some substances can act on the healthy ectoderm cells in such a way as to cause the release of their stores of previously inactivated evocator. If this release of the evocator occurs while the ectoderm still retains its competence then the cells would presumably respond by neuralization to the active evocatory stimulus now released within them. This is probably what happens when explants of competent ectoderm are exposed to artificial evocators in solution, where all the cells may respond by neuralization without any of them showing signs of necrosis (Shen, 1942). There is a certain similarity between an artificial evocator acting indirectly in this way and the normal evocator, since we know that when the latter diffuses from the mesoderm into the ectoderm it not only causes neuralization but also stimulates the ectoderm to produce more evocator substance, whose activity is exhibited in the phenomenon of 'homoiogenetic induction', i.e. induction of neural tissue by implants of neural tissue. We may speak of this as a *physiological activation* of the bound evocator, as opposed to the *cytolytic activation* which occurs when definitely lethal conditions, such as heat coagulation or mechanical disruption, are applied.

It is, then, very possible that the carcinogen evocators cause a physiological activation of the previously inactive evocator. If so, it would be most simple to suppose that the locus of this activation is at the lipochondria, where the hydrocarbon can be seen to accumulate. We have seen that after treatment with benzpyrene the lipochondria rather rapidly break down, their lipide constituents running together to form larger liposomes. Holtfreter (1946*c*) has shown that a similar process occurs in normal development and is noticeable first in the archenteron roof, that is, in the tissue in which the evocator first becomes active. It is tempting to suggest that this breakdown of the lipochondria is actually the process of liberation of free evocator from an inactive complex which was first adumbrated some dozen years ago (Waddington, Needham, and Brachet, 1936).

It should be noted that this suggestion does not entirely conflict with Brachet's hypothesis as to the importance of ribonucleoprotein granules in the induction process. Brachet (1943) has shown that such granules increase in number in the archenteron roof as it invaginates, that is, at the same time and place as the lipochondria break down. Now the lipochondria certainly consist not only of lipide but also contain protein material, probably arranged as an

envelope covering the lipide core (Holtfreter, 1946*a*). There is as yet no convincing evidence that this envelope is nucleoprotein in nature, but we have found that the lipochondria stain, albeit rather faintly, with pyronine and lose their affinity for that stain after digestion in crude ribonuclease. It is, therefore, by no means impossible that the appearance of Brachet's granules is not merely correlated with, but is an actual consequence of the breakdown of the lipochondria, and it is possible to suppose that they are in fact the liberated evocator.

Holtfreter (1948) drew attention to the fact that during cytolysis, when the evocator is known to become liberated, the lipochondria can be seen to break down and their lipide constituents to become free. It is clear that he considered that the correlation between the two phenomena might be significant. In his discussion of the matter, however, he does not pursue it very far; instead he turns to a mention of the cytoplasmic basiphilic granules, which he derives, not from the protein part of the lipochondria, but by an 'Entmischung' from the originally clear cytoplasm. He suggests that both the mobilization of the lipides from the lipochondria and the precipitation of granules in the cytoplasm are secondary consequences of changes in the cell surface, these being of such a kind as to lead to increased permeability. It appears to us that the demonstration that the carcinogen evocators become accumulated directly at the lipochondria, and not at the cell surface, favours the simpler hypothesis advanced above, that the evocator-liberation takes place directly at the lipochondria.

Further work is required, however, before this can be accepted as more than one of a number of possibilities. It still remains possible that it is the lipoid component of the lipochondria which is the active evocator. And finally we cannot yet afford to overlook the possibility that the carcinogens are acting not as activators, either cytolytic or physiological, but as direct evocators which are chemically sufficiently allied to the natural substance to simulate its action on the ectoderm cell. What has been gained in this investigation is not a final resolution of these various alternatives, but a strong suggestion that the lipochondria are the site of evocator activation and possibly of evocator action.

Summary

Cells from explants of newt gastrulae which had been cultured in Holtfreter's standard solution containing the carcinogen 3:4-benzpyrene rendered soluble by caffeine were examined for fluorescence by ultra-violet light. The lipoid constituents of the cells, lipochondria and liposomes, showed the bright blue fluorescence of benzpyrene in molecular solution and there was also occasionally a structureless blue fluorescence round the nucleus corresponding perhaps to the so-called 'Golgi Apparatus'. The yolk platelets were slightly fluorescent but this was probably due to the associated lipides and it largely disappeared in moribund preparations, where the lipochondria became detached from the platelets. The nucleus showed no fluorescence, nor was a fluorescent nucleolus observed. It could be shown that the pyronine-

staining cytoplasmic granules of ribonucleoprotein (microsomes) were not fluorescent and so probably do not have any affinity for the carcinogen. The very small fluorescent granules in Brownian movement that had previously been looked upon as ribonucleoprotein microsomes are in fact small liposomes and do not stain with pyronine.

It is suggested that the evocating power of the carcinogenic hydrocarbons is probably not due to *cytolytic activation* of the evocator, by the killing of some cells in the exposed ectoderm. It may be by *physiological activation*, operating through a specific action on the lipochondria, leading to their breakdown into liposomes and the release of active evocator substance. Some evidence is produced which suggests that the lipochondria contain ribonucleoprotein, and this fraction of them may constitute the active evocator; this would be congruent with the theories of Brachet (1947). It cannot yet be excluded, however, that the hydrocarbons act as *direct evocators*, simulating the effects of the naturally occurring substance.

The observation that benzpyrene is not accumulated by nucleoprotein makes it likely that the mutagenic activity of the carcinogens is indirect.

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